Biochemical Pharmacology, Vol. 59, pp. 131–139, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00305-6

Ligand-binding Affinity of the Type 1 and 2 Inositol 1,4,5-trisphosphate Receptors: Effect of the Membrane Environment

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ABSTRACT. The inositol 1,4,5-trisphosphate ($InsP_3$) receptor is essential for Ca^{2+} release from intracellular stores. There are three $InsP_3$ receptor types which are targets for several types of regulation. Ca^{2+}, phosphorylation, and protein–protein interactions may contribute to the complex pattern of the Ca^{2+} signal in stimulated cells. Furthermore, the 3 receptor types could have different affinities for $InsP_3$. We compared the affinities of the type 1 receptor from the cerebellum with the liver type 2 receptor both in their membrane environment and after isolation by immunoprecipitation. Measurements of [^{3}H]$InsP_3$ binding in a cytosol-like medium revealed that the K_d of the liver receptor (45 ± 5 nM, N = 14) was higher than the K_d of the cerebellar receptor (28 ± 3 nM, N = 9). Solubilization and immunopurification of the liver $InsP_3$ receptor resulted in a 10-fold increase in its affinity for $InsP_3$. The affinity of the cerebellar receptor did not change under these conditions. Therefore, the extraction of the liver and the cerebellar receptors from their membrane environments induced an inversion of their relative affinities. Treatment of liver membranes with low concentrations of detergents also increased the affinity for $InsP_3$ binding. These data indicate that the type 1 and the type 2 $InsP_3$ receptors have different affinities for $InsP_3$ and that the properties of the type 2 receptor are strongly regulated by hydrophobic interactions within its membrane environment. BIOCHEM PHARMACOL **59**;2:131–139, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. inositol 1,4,5-trisphosphate receptor; calcium release channel; binding affinity; immunoprecipitation, liver; cerebellum

The Ca^{2+} signal observed in target cells in response to extracellular stimuli follows a highly complex spatio-temporal pattern which differs according to the cell type and to the stimulus [1]. The complexity arises from the diversity of the molecular elements involved in Ca^{2+} homeostasis. One important step in the Ca^{2+} signaling pathway is Ca^{2+} release from the endoplasmic reticulum through a Ca^{2+} channel activated by $InsP_3$† [1]. At least 3 genes are responsible for the synthesis of the different $InsP_3R$ subunits and alternative splicing of the mRNA gives rise to different isoforms of the type 1 subunit [2–5]. $InsP_3R$ forms tetrameric structures comprised of different subunits, resulting in a great number of different combinations of functional $InsP_3R$ complexes [6, 7]. Therefore, the $InsP_3$-induced Ca^{2+} release in a given cell type, which first depends on $InsP_3$ binding to its receptors, depends on numerous variables, including the type(s) of receptor present in the cells and the type of regulation by Ca^{2+}, phosphorylation, and protein–protein interactions (for review, see [8–10]).

The different types of receptors are co-expressed in various tissues, but there is some tissue specificity in their distribution. The $InsP_3R$-1 receptor is abundant in the cerebellum and the brain, $InsP_3R$-2 is found predominantly in the liver, lung, testis, and spleen, and the $InsP_3R$-3 in the intestine and the pancreatic islets [4, 5, 11]. This suggests that the different types of $InsP_3R$ have different intracellular functions. Indeed, they may have different affinities for $InsP_3$ with a relative order of specificity of type 2 > type 1 > type 3 [11]. The different receptor types are therefore presumably differently responsive to $InsP_3$ and the response may also vary according to tissue or intracellular location. However, the K_ds determined in various tissues and cell lines give no clear evidence of a correlation between the expression of a given receptor type and its affinity. The determination of the apparent affinity of the $InsP_3R$-3 varied over a 100-fold range [5, 12, 13]. In some experiments, it was found that the $InsP_3R$-2 had a higher affinity than the $InsP_3R$-1 [11, 14–16], while in another study it was reported that the order was $InsP_3R$-1 > $InsP_3R$-2 [17]). These discrepencies could be due to the different experi-

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† *Abbreviations:* $InsP_3$, inositol 1,4,5-trisphosphate; and $InsP_3R$, $InsP_3$ receptor.

Received 12 April 1999; accepted 8 July 1999.

mental protocols used for K_d determinations, as the binding properties of the $InsP_3R$ depend on the pH, the ionic strength or the presence of nucleotides [18], and possibly other factors such as the red-ox status of the cell, the Ca^{2+} concentration of the incubation medium, or protein–protein interactions.

The liver and the cerebellum are representative of several typical characteristics of the $InsP_3R$. In the cerebellum, more than 90% of the $InsP_3R$ are type 1, whereas in liver cells 80% are type 2, 20% are type 1, and there are trace amounts of type 3 receptor [19, 20]. The receptors in these tissues are differently regulated: Ca^{2+} decreases the $InsP_3$ binding to the cerebellar receptor [21] and the liver receptor is transformed into a high-affinity desensitized state by Ca^{2+} [22]. The receptors are also differently distributed within the 2 cell types: in cerebellar Purkinje cells, $InsP_3R$ is located on endoplasmic reticulum membranes and is widely distributed throughout the cytoplasm (see [23]), whereas in hepatocytes $InsP_3R$-1 is mainly found in specialized subregions of the endoplasmic reticulum located at the periphery of the cell [24]. We compared the binding properties of the cerebellar $InsP_3R$-1 and the liver $InsP_3R$-2, in their membrane environment and after isolation, in a medium which mimics the cytosol. The membranous $InsP_3R$-2 had a lower affinity than the $InsP_3R$-1. The $InsP_3R$-2 is regulated by hydrophobic interaction and its K_d was substantially decreased by isolation from its membrane environment.

MATERIALS AND METHODS

Materials

[^{3}H]$InsP_3$ (17–21 Ci/mmol) was obtained from DuPont New England Nuclear, $InsP_3$ from Calbiochem, and all other reagents from Sigma or Boehringer Mannheim. The antibodies against the 14 C-terminal residues or against residues 337–349 of the $InsP_3R$-1 have been described previously [25]. Antibodies against a synthetic peptide corresponding to the 16 C-terminal residues (2686–2701) of the $InsP_3R$-2 were prepared by Covalab and have been described previously [26].

Preparation of the Membrane Fractions

Cerebella or lungs from female Wistar rats (200–250 g) were homogenized with an Ultra-Turrax in the ice-cold homogenization medium containing 250 mM sucrose, 5 mM HEPES pH 7.4, 1 mM EGTA, supplemented with 1 mM dithiothreitol and a protease inhibitor cocktail constituted of 0.4 mM phenylmethylsulfonyl fluoride and 10 μg/mL leupeptin, 10 μM pepstatin, 2 mM benzamidine, 5 μg/mL aprotinin, 50 μg/mL trypsin inhibitor, and 1 μg/mL 1,10-phenanthroline as described previously [25]. The homogenates were centrifuged for 5 min at 1500 *g*. The supernatants were collected and centrifuged for 30 min at 50,000 *g*. The pellets were washed and resuspended at 4 mg protein/mL in washing medium containing 250 mM sucrose, 25 mM HEPES pH 7.4, supplemented with 1 mM dithiothreitol and the protease inhibitor cocktail. Livers from female Wistar rats (200–250 g) were homogenized with a Dounce homogenizer in the ice-cold homogenization medium. The homogenate was centrifuged for 10 min at 1500 *g*, yielding the low-speed pellet. The plasma membrane fraction, enriched in $InsP_3R$, was prepared by Percoll gradient centrifugation of the low-speed pellet as previously described [22]. The membranes obtained were washed and resuspended in the washing medium. In some experiments, the membrane fractions were resuspended in the washing medium to give a protein concentration of 1 mg/mL and incubated in the presence of different detergents at the indicated concentrations for 30 min at 4°. The mixture was then centrifuged for 60 min at 100,000 *g*. The pellet was resuspended in the binding medium mimicking the ionic composition of the cytosol and containing 110 mM KCl, 20 mM NaCl, 25 mM HEPES pH 7.4, 1 mM NaH_2PO_4, 1 mM EDTA, supplemented with 1 mM dithiothreitol and the protease inhibitor cocktail, and used in [^{3}H]$InsP_3$-binding experiments. Alkaline treatment of membranes was performed by incubation for 10 min in the homogenization medium adjusted to pH 11 by the addition of NaOH. The membranes were then centrifuged for 20 min at 20,000 *g*, washed in washing medium, centrifuged again at 20,000 *g*, resuspended in the binding medium, and used in [^{3}H]$InsP_3$-binding experiments. Hepatocytes were prepared as previously described [27]. They were washed and resuspended at 4×10^6 cells/mL in the binding medium and permeabilized at 4° by the addition of 80 μg/mL saponin.

Immunoprecipitation of the Solubilized $InsP_3R$

Membrane fractions were washed, resuspended to give a protein concentration of 4 mg/mL in the binding medium, and incubated for 30 min on ice in the presence of 1.5% (w/v) Triton X-100. The mixtures were centrifuged for 1 hr at 100,000 *g*. The supernatants were dialyzed 3 times for 30 min against 500 mL of the binding medium containing 0.1% Triton X-100. The resulting solubilized membrane proteins were incubated overnight at 4° with affinity-purified antibodies directed against the C-terminal end of the $InsP_3R$-1 or with antiserum against the C-terminal end of the $InsP_3R$-2 diluted 1/25. Samples (1 mL) were incubated with 100 μL of Sepharose-Protein A beads for 2 hr and centrifuged at 1500 *g* for 5 min. The pellet was washed five times with the binding medium containing 0.1% Triton X-100. The immunoprecipitated material was used directly for [^{3}H]$InsP_3$ binding studies or removed from the Sepharose beads by boiling the sample in an equal volume of electrophoresis sample buffer and analyzed by Western blotting.

SDS-PAGE and Western Blotting

Membrane fractions were resuspended in SDS sample buffer under reducing conditions [28]. SDS–PAGE was performed on 4–10% polyacrylamide gradient gels and the separated

proteins were electrotransferred to a Hybond C-Super nitrocellulose membrane (Amersham) [29]. The blots were saturated with 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween 20 in PBS for 1 hr at 37°. The blots were then incubated overnight at 4° with appropriate antibodies at the indicated dilution in PBS containing 2.5% (w/v) non-fat dry milk and 0.1% Tween 20 (v/v). After 5 washes, the nitrocellulose membranes were incubated for 30 min at room temperature with peroxidase-conjugated anti-rabbit immunoglobulin G goat antibodies (1:2000) (Diagnostic Pasteur). Blots were then washed 5 times and developed with the enhanced chemiluminescence (ECL) Western blotting system using Hyperfilm (Amersham).

Equilibrium [^{3}H]$InsP_3$-binding Studies

Membrane fractions (100–500 μg) or immunoprecipitated material (10 μL) were incubated on ice in 500 μL of the binding medium containing 1 mg/mL BSA and supplemented with 1 nM [^{3}H]$InsP_3$. Non-specific binding was determined in the presence of 5 μM $InsP_3$. After a 6-min incubation, 400 μL of the sample was layered onto a Whatman GF/C glass fibre filter and washed with 1 mL of ice-cold medium consisting of 250 mM sucrose, 10 mM Na_2HPO_4 and adjusted to pH 8 to minimize the dissociation of the ligand–receptor complex. Radioactivity retained on the filter was counted by scintillation spectrometry. Binding of [^{3}H]$InsP_3$ to solubilized receptor was assayed in 500 μL of the binding medium containing 0.1% Triton X-100 and 1 nM [^{3}H]$InsP_3$ for 6 min at 2°. The reaction was stopped by the addition of 20 μL of γ-globulin at 50 mg/mL and 500 μL of 30% polyethylene glycol. After a 5-min incubation, the mixture was centrifuged at 14,000 *g* for 5 min. The pellet was resuspended in Soluene and counted by scintillation spectrometry.

RESULTS

Properties of the Membranous Receptor

We performed homologous competition [^{3}H]$InsP_3$-binding experiments on liver membranes and cerebellar membranes in parallel in a medium mimicking the ionic composition of the cytosol. Cerebellum contains mainly type 1 receptor and the liver mainly type 2 receptor [6, 19, 20]. The affinity of these two $InsP_3R$ types were thus compared in the same physiological medium in the absence of known regulators (Fig. 1). The data for both fitted a single-site model and the inhibition curve for the liver membranes was shifted to the higher $InsP_3$ concentrations as compared to the curve for the cerebellar membranes. Mean values given in Table 1 indicated that the liver receptor (K_d = 45 ± 5 nM, N = 14) had a significantly lower affinity than the cerebellar receptor (K_d = 28 ± 3 nM, N = 9). The measurement of [^{3}H]$InsP_3$ binding to saponin-permeabilized hepatocytes gave a K_d of 34 nM (Table 1), which was very close to the value found in the liver membrane fraction. Therefore, the liver membrane fraction used in the present work behaved like the whole hepatocytes. Our homologous competition binding experiments favor the labeling of the high-affinity sites [30], and consequently, our experiments would detect the type 2 receptor present in liver membranes if it were in a high-affinity conformation. This suggests that the liver type 2 receptor did not behave like the recombinant soluble

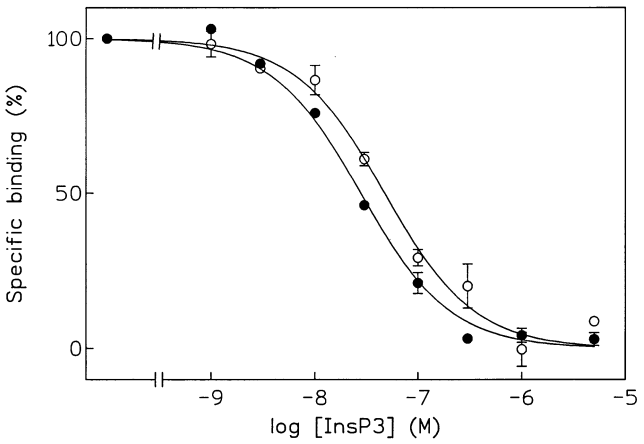

FIG. 1. Inhibition of [^{3}H]$InsP_3$ binding to liver and cerebellar membranes by unlabeled $InsP_3$. The liver (540 μg, ○) or cerebellar (122 μg, ●) membranes were incubated in the binding medium containing 1 nM (11,000 cpm) [^{3}H]$InsP_3$ and the indicated concentrations of unlabeled $InsP_3$. Data points are means of triplicate determinations in 1 experiment. Values for specific binding in the absence of inhibitor were 432 cpm and 1228 cpm for liver and cerebellum, respectively. The analysis of the binding data indicated that slope factors were 0.99 and 1.15, K_ds were 47 nM and 24 nM, and B_{max} were 3.4 pmol/mg and 27 pmol/mg for liver membranes and cerebellar membranes, respectively.

TABLE 1. [^{3}H]$InsP_3$-binding parameters of the type 1 and type 2 $InsP_3$ receptors

Material	K_d (nM)	Slope factor	N
Cer. memb.*	28 ± 3	1.1 ± 0.1	9
Cer., IP1C	23 ± 2	1.1 ± 0.05	4
Cer., IP2C	1.6	0.91	2
Liver memb.*	45 ± 5	0.85 ± 0.05	14
Perm. hepatocytes	34	0.8	2
Liver, solub.	3.3	0.93	1
Liver, unsolub.	3.6	0.95	1
Liver, IP1C†	3.9 ± 1.2	0.84 ± 0.1	9
Liver, IP1N	7	1.4	1
Liver, IP2C†	5.5 ± 0.8	0.97 ± 0.06	7
Lung memb.	46	0.98	1
Lung, IP2C	1.8	0.99	1

Cerebellar (Cer.), liver, or lung membranes were treated in the presence of 1.5% Triton X-100 and centrifuged at 100,000 *g*, and the supernatant (solub.) and the pellet (unsolub.) were recovered for [^{3}H]$InsP_3$-binding measurements. $InsP_3R$ was immunoprecipitated from the solubilized material with antibodies against either the C-terminal end of the $InsP_3R$-1 (IP1C) or the $InsP_3R$-2 (IP2C), or the 337–349 residues of the $InsP_3R$-1 (IP1N). [^{3}H]$InsP_3$ binding was measured as indicated. Binding parameters are means ± SEM of values determined in N independent experiments.

*K_ds for cerebellar membranes and liver membranes are significantly different ($P < 0.01$).

†K_ds for the immunoprecipitated liver receptor are significantly different from the K_d for the liver membranes ($P < 0.001$).

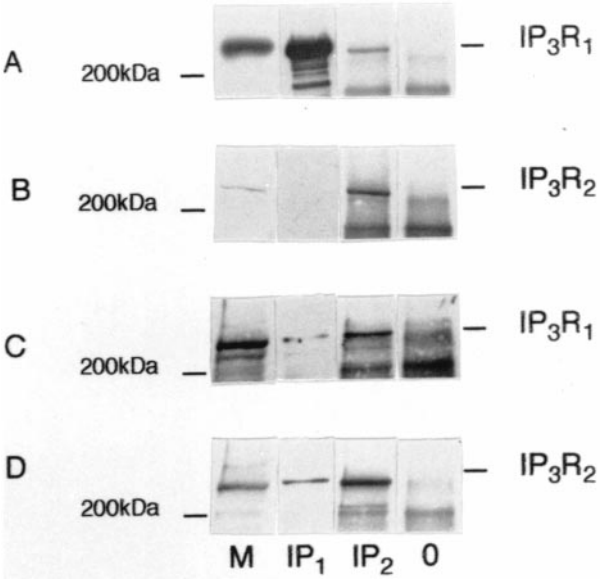

FIG. 2. Immunoprecipitation of the type 1 and the type 2 $InsP_3R$ from cerebellar and liver membranes. Cerebellar membranes (A and B) and liver membranes (C and D) were solubilized and incubated with affinity-purified antibodies raised against the 14 C-terminal amino acids of the $InsP_3R$-1 (IP_1) or with an antiserum against the 16 C-terminal amino acids of the $InsP_3R$-2 (IP_2) or with non-specific immunoglobulins (0). Immune complexes were collected with Sepharose-Protein A beads. Equal amounts of immunoprecipitated material were separated by SDS–PAGE (4–10% gradient) and then electrotransferred to a nitrocellulose membrane. Membranes (M) from cerebellum (15 μg, A and B) or from liver (50 μg, C and D) were blotted as control. The blots were developed with antibodies against the $InsP_3R$-1 (A and C) or against the $InsP_3R$-2 (B and D) and with peroxidase-conjugated anti-rabbit immunoglobulin G goat antibodies (1:2000). The development of each blot was adjusted in order to maximize the labeling with each antibody. Therefore, the proportion of $InsP_3R$-1 and $InsP_3R$-2 cannot be compared between the different samples. Size marker positions (in kilodaltons) are indicated on the left.

N-terminal binding domain of the $InsP_3R$-2, which had a higher affinity that the analogous $InsP_3R$-1 N-terminal-binding domain [3].

Properties of the Immunoprecipitated Receptor

In the next experiments, we extracted and immunoprecipitated the liver and cerebellar receptors to compare their intrinsic binding properties outside their membrane environment. Immunoprecipitation with specific antibodies is rapid and the ligand-binding assay can be easily performed directly on the immunoprecipitated material without any other treatment [31]. This protocol also enables comparison of the binding properties of the purified receptor with those of the receptor in the membrane under the same experimental conditions. Liver membrane proteins were solubilized in Triton X-100 and the anti-$InsP_3R$-2 antibody used for immunoprecipitation. Western blotting analysis of the immunoprecipitated material revealed the presence of both $InsP_3R$-2 and $InsP_3R$-1 (Fig. 2, C and D). Similarly, anti-$InsP_3R$-2 antibody immunoprecipitated both $InsP_3R$ types (Fig. 2, C and D). This confirms the presence of heterotetramers in the liver. Using protein extracts from the cerebellar membranes, only $InsP_3R$-1 was detected in the material immunoprecipitated with the anti-$InsP_3R$-1 antibody, and the anti-$InsP_3R$-2 antibody immunoprecipitated both $InsP_3R$-1 and $InsP_3R$-2 (Fig. 2, A and B). This indicates that the cerebellum contains heterotetramers made up of $InsP_3R$-1 and $InsP_3R$-2 monomers. $InsP_3R$-1 has been reported to be found mainly in neurons and granule cells and the $InsP_3R$-2 in astrocytes [32–34]. It can be assumed that heterotetramers containing $InsP_3R$-2 came from the astrocytes and those containing $InsP_3R$-1 mainly from neuronal cells. The cerebellar receptor immunoprecipitated with the antibody against $InsP_3R$-1 specifically bound [^{3}H]$InsP_3$. Figure 3A shows the inhibition curves of [^{3}H]$InsP_3$ binding to cerebellar membranes and to receptor immobilized to Protein A-Sepharose beads: the mean IC_{50}s

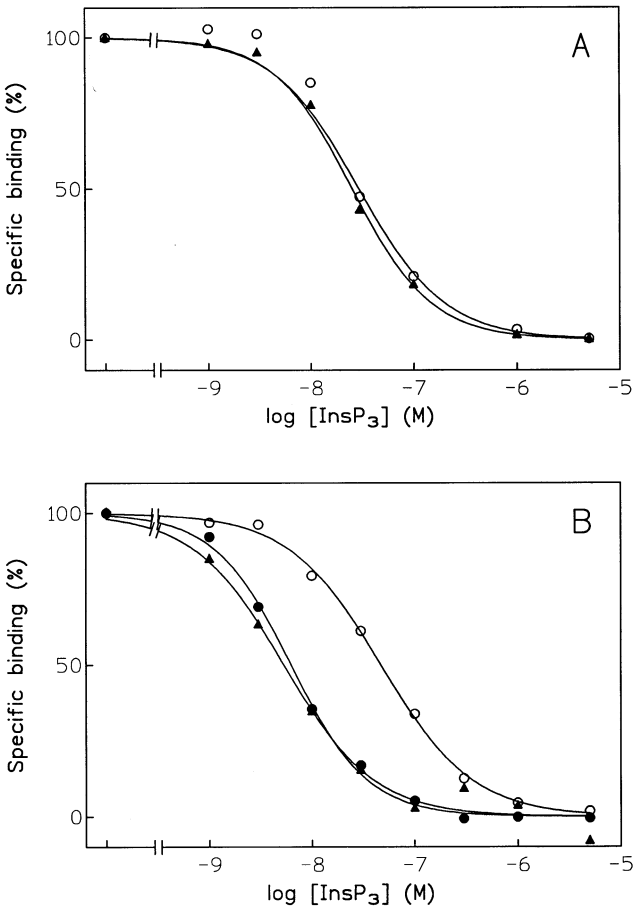

FIG. 3. Comparison of $InsP_3$-binding properties of purified and membranous $InsP_3R$ from cerebellum (A) or liver (B). (A) Cerebellar membranes (○) or $InsP_3R$ immunoprecipitated with antibody against the C-terminal end (▲) of the type 1 receptor were incubated as indicated in Fig. 1. (B) Liver membranes (○) or $InsP_3R$ immunoprecipitated with antibody against the C-terminal end of the $InsP_3R$-2 (●) or against the C-terminal end of the $InsP_3R$-1 (▲) were incubated as indicated in Fig. 1. Data points are means of triplicate determinations of 1 experiment. Mean values for binding parameters are given in Table 1.

were 28 ± 3 nM (N = 9) and 23 ± 2 nM (N = 4), respectively. The difference between the K_ds, determined in parallel in 4 experiments, was not significant according to the paired *t*-test. These data indicate that the binding properties of the type 1 receptor of the rat cerebellum were not modified upon solubilization and immunoprecipitation.

The liver receptor immunoprecipitated with the anti-$InsP_3R$-2 antibody also bound [^{3}H]$InsP_3$. Competition binding assays were conducted in parallel with the immunoprecipitated receptor and the liver membranes, and the 2 curves were satisfactorily described by a single site model (Fig. 3B). The K_d of the immunoprecipitated receptor (5.5 ± 0.8 nM, N = 7) was significantly lower than the K_d of the membrane fraction (45 ± 5 nM, N = 14) (Table 1). The measurement of the kinetics of dissociation of the [^{3}H]$InsP_3$ from the immunoprecipitated liver receptor indicated that the $InsP_3R$ was in a high-affinity state with a rapid dissociation rate ($t_{1/2} < 1$ min, not shown). This was different from the high-affinity state induced by micromolar concentrations of Ca^{2+}, in which the dissociation rate is much slower ($t_{1/2} > 60$ min) [27]. We also used antibodies against the type 1 receptor to immunoprecipitate the liver $InsP_3R$. If we assume that the liver membranes contained 80% $InsP_3R$-2 and 20% $InsP_3R$-1 and that the tetramers are associated randomly, it can be calculated [7] that 60% of the tetramers are heterotetramers. These heterotetramers can be immunoprecipitated by anti-$InsP_3R$-1 antibodies and would contain 66% $InsP_3R$-2. The material immunoprecipitated with antibodies directed against the C-terminal end of the type 1 receptor bound $InsP_3$ with a high affinity (Fig. 3B), with a K_d of 3.9 ± 1.2 nM (N = 9). We also used another antibody against the residues 337–349 localized in the N-terminal domain of the type 1 receptor. Again, the immunoprecipitated receptor bound $InsP_3$ with a high affinity (K_d = 7 nM, Table 1). We treated the liver membrane with Triton X-100 and separated the soluble and insoluble material. [^{3}H]$InsP_3$ binding to solubilized proteins was measured by the polyethylene glycol precipitation assay and [^{3}H]$InsP_3$ binding to insoluble material was measured by the filtration method. The K_ds of the $InsP_3$-binding sites of the solubilized receptor and of the insoluble material were nearly identical (3.3 and 3.6 nM, respectively, Table 1). Thus, the modification of the properties of the liver receptor following isolation from the membrane and immunoprecipitation is neither due to a selection of a special population of receptors during the solubilization and the purification protocol nor to the interaction of the antibodies with the receptor, changing its conformation.

Effect of Various Inhibitors on [^{3}H]$InsP_3$ Binding to $InsP_3R$-2

Heterologous competition experiments were performed using membranes prepared from rat liver, and receptor immunoprecipitated from liver membrane extract. The order of specificity with the membranes was: Ins(1,4,5)P_3 > glycerophospho-Ins(4,5)P_2 = Ins(2,4,5)P_3 > Ins(1,3,4,5)P_4 ≫ Ins(1,3,4)P_3, and heparin efficiently inhibited [^{3}H]$InsP_3$ binding (Table 2). The order of potency of the various inhibitors for the immunoprecipitated liver $InsP_3R$ was the same as that for the membranous $InsP_3R$, but the IC_{50} for each inhibitor, including heparin, was 3- to 6-fold lower. This indicates that the shift in affinity resulting from immunoprecipitation is not observed only with Ins(1,4,5)P_3.

TABLE 2. Specificity of [^{3}H]$InsP_3$ binding to liver membranes and to immunoprecipitated liver type 2 receptor

	IC_{50} (μM)	
Inhibitor	**Liver memb.**	**$InsP_3R$-2**
Ins(1,4,5)P_3	0.035	0.006
Ins(1,3,4,5)P_4	3.4	0.86
Ins(2,4,5)P_3	0.43	0.14
GPIns(4,5)P_2	0.49	0.086
Ins(1,3,4)P_3	>100	11
Heparin	8.5 μg/mL	2.1 μg/mL

EC_{50}s are means of values determined in 2 experiments.

Tissue Specificity of the Properties of the $InsP_3R$-2

We found that the liver material immunoprecipitated with antibodies against either the $InsP_3R$-1 or the $InsP_3R$-2 contained a large amount of $InsP_3R$-2 (see Fig. 2D) and had a higher affinity than the membranous receptor, whereas those of the cerebellar $InsP_3R$-1 were not modified upon immunoprecipitation. We wondered whether this different behavior was due to the properties of the receptor types or to their tissue distribution. Cerebellar membranes contained a small but detectable amount of $InsP_3R$-2, which was immunoprecipitated by anti-type 2 antibodies (Fig. 2B). [^{3}H]$InsP_3$-binding assays indicated that the immunoprecipitated cerebellar type 2 receptor had a high affinity (K_d = 1.6 nM), consistent with that of the liver receptor. We also studied the properties of the $InsP_3R$-2 in the lung, a tissue in which the proportion of $InsP_3R$-2 is high [20]. The K_d for $InsP_3$ binding to the membrane fraction was 46 nM and 1.8 nM to the receptor immunoprecipitated with anti-type 2 receptor antibodies (Table 1). These results indicate that the shift in affinity after solubilization and immunoprecipitation was found when a high proportion of $InsP_3R$-2 was present in the preparation. This indicates that the type 2 receptor, whatever its tissue origin, is maintained in a low-affinity conformation within the membrane and has a high affinity when extracted with detergent.

Influence of the Membrane Environment

Solubilization of the liver membrane proteins might have separated the $InsP_3R$ from other components necessary to maintain the properties of the receptor. To test this, we treated the liver membranes with low concentrations of Triton X-100 to modify the interactions with lipids or with membrane proteins. Competition binding experiments re-

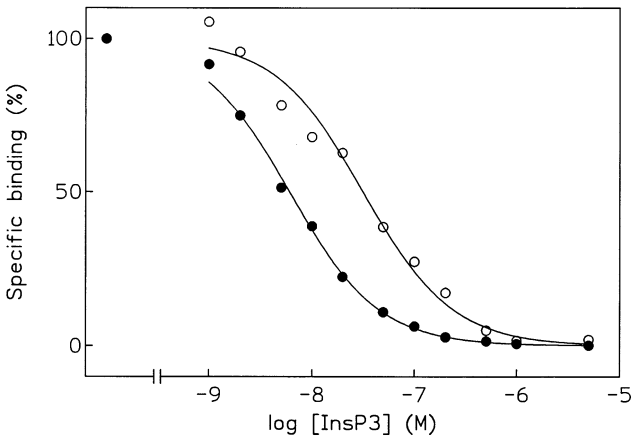

FIG. 4. Effect of Triton X-100 on [^{3}H]InsP$_3$ binding to liver membranes. Liver membrane fraction was preincubated without (○) or with 0.1% Triton X-100 (●) and centrifuged at 100,000 *g*. [^{3}H]InsP$_3$ binding to the pellet fraction was assayed after resuspension in the binding medium with the indicated InsP$_3$ concentrations. Data points are means of triplicate determinations of 1 experiment repeated twice.

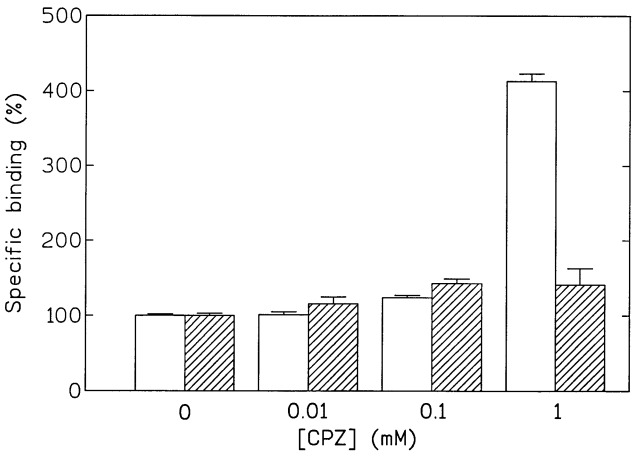

FIG. 5. Effects of chlorpromazine on [^{3}H]InsP$_3$ binding to liver membranes and cerebellar membranes. Liver membranes (open bars) or cerebellar membranes (hatched bars) were incubated for 6 min in the binding medium in the presence of 1 nM [^{3}H]InsP$_3$ and the indicated concentrations of chlorpromazine (CPZ). The reaction was stopped as described in Materials and Methods. Specific binding was 870 ± 16 cpm for liver membranes and 1360 ± 37 cpm for cerebellar membranes. Data points are means ± SEM of triplicate determinations in 1 experiment representative of 3.

vealed that pretreatment of the membranes with 0.1% Triton X-100 decreased the K_d from 33 nM to 5.6 nM (Fig. 4). Western blotting of the pretreated membranes indicated that they contained both InsP$_3$R-1 and InsP$_3$R-2 in about the same proportion as the untreated membranes (not shown). [^{3}H]InsP$_3$ binding to liver membranes was also increased after pretreatments with low concentrations of several other non-ionic or zwitterionic detergents. None of these pretreatments increased [^{3}H]InsP$_3$ binding to cerebellar membranes (Table 3); instead, some decreased binding to cerebellar membranes, suggesting that InsP$_3$R could be partly solubilized or inactivated. Higher concentrations than those indicated in Table 3 decreased InsP$_3$ binding to liver and cerebellar membranes either by solubilizing the receptor or by inactivation. The ionic detergents deoxycholate or SDS inhibited [^{3}H]InsP$_3$ binding to membranes even at low concentrations. These data confirm that the modifications of the binding properties of the isolated liver receptor were not the result of a selection of a particular type of InsP$_3$R. In addition, these results indicate that the disruption of the membrane by detergents substantially modified the affinity of the liver receptor to InsP$_3$, but did not change the properties of the cerebellar receptor.

TABLE 3. Effects of detergents on [^{3}H]InsP$_3$ binding to liver membranes and cerebellar membranes

	Specific binding (%)	
Addition	**Liver**	**Cerebellum**
no	100 ± 5	100 ± 1
Triton X-100 (0.05%)	204 ± 7*	58 ± 2*
Triton X-100 (0.1%)	277 ± 9*	62 ± 1*
CHAPS (0.1%)	136 ± 3†	96 ± 1*
CHAPS (0.5%)	248 ± 4‡	93 ± 2
Zwittergent 3–14 (0.01%)	146 ± 4†	108 ± 1
Thesit (0.01%)	116 ± 1‡	105 ± 4
Thesit (0.1%)	194 ± 7*	116 ± 4
Thesit (1%)	250 ± 3*	107 ± 4
n-Octylglucoside (0.01%)	118 ± 3‡	102 ± 1

Membranes were preincubated with the indicated detergent concentrations as indicated in Materials and Methods. They were incubated for 6 min in the binding medium in the presence of 1 nM [^{3}H]InsP$_3$. Results are means ± SEM of triplicate determinations for 2 experiments. The specific binding in the control liver and cerebellar membranes were 62 fmol/mg and 862 fmol/mg, respectively.

*$P > 0.001$, †$P < 0.01$, ‡$P < 0.05$ as compared to control.

These effects of the detergents on the properties of the liver InsP$_3$R suggest that hydrophobic interactions could be involved in the regulation of the affinity of the receptor. We also tested the action of other classes of molecule known to be involved in hydrophobic interaction. Chlorpromazine (1 mM) induced a 4-fold increase in the binding of [^{3}H]InsP$_3$ to liver membranes and had a moderate effect on the binding to cerebellar membranes, as did detergents (Fig. 5). This effect is unlikely to be related to its anti-calmodulin properties, because it occurs at much higher concentrations than those required to inhibit calmodulin and is independent of the presence of Ca^{2+}. Chlorpromazine is also known as a local anesthetic able to modulate the other intracellular Ca^{2+} channel, the ryanodine receptor [35]. Procaine (3 mM), tetracaine (3 mM), caffeine (5 mM), and ethanol (2%), which have anesthetic properties, did not reproduce the effects of chlorpromazine and detergents on the binding properties of the InsP$_3$R (not shown). This indicates that the effects of detergents and of chlorpromazine are not related to the action of anesthetics. These data strongly suggest that liver InsP$_3$R-2 is regulated by hydrophobic interactions with a membranous component.

It is possible that the treatments with the detergents

modified interactions with integral membrane components and were responsible for the modification of the binding properties of the $InsP_3R$. The $InsP_3R$ can also be regulated by interaction with peripheral proteins, including actin, ankyrin, and FK506-binding protein [31, 36, 37]. Some of these interactions may contribute to regulate the $InsP_3R$ function or properties. To test whether the liver receptor is regulated by integral or peripheral components, we treated the membrane fraction with media known to extract peripheral membrane proteins. We previously found that such a treatment suppressed the effects of Ca^{2+} on $InsP_3$ binding to liver membranes [26]. Pretreatment of membranes in a medium with 1 M KCl had no effect on the binding properties of the receptor (not shown). Similarly, pretreatment of the liver membranes in a medium adjusted to pH 11 had little effect on the receptor properties, most of the receptors remaining in a low-affinity state with a K_d of 56 nM (not shown). This indicates that the increased affinity of the liver receptor following detergent treatment is not the result of extraction of a protein in electrostatic interaction with the membrane.

DISCUSSION

We have found that $InsP_3$ bound to liver membranes with a lower affinity than to cerebellar membranes. The cerebellum contains a high proportion of $InsP_3R$-1 and the liver a high proportion of $InsP_3R$-2. Therefore, it can be concluded that $InsP_3$ binds to liver $InsP_3R$-2 with a lower affinity than to cerebellar $InsP_3R$-1. Our study of the binding of $InsP_3$ to $InsP_3R$-1 and $InsP_3R$-2 in membrane fractions and after immunoprecipitation shows that the affinity of the $InsP_3R$ depends on the receptor type and that $InsP_3R$-2 is regulated by its membrane environment. It has previously been observed that detergent solubilization of the $InsP_3R$ from hepatocytes stimulated binding of the ligand [38]. We now demonstrate that this stimulation is due to an increase in the affinity of the type 2 receptor. Indeed, the presence of the high-affinity $InsP_3$ binding site is correlated with the presence of $InsP_3R$-2 in the immunoprecipitated material as well from a tissue of the central nervous system such as the cerebellum and from peripheral tissues such as the liver or the lung. Western blotting experiments indicated that the $InsP_3R$-2 immunoprecipitated from liver or cerebellar membranes also contained $InsP_3R$-1 co-immunoprecipitated as heterotetramers (Fig. 2). Since the immunoprecipitated cerebellar $InsP_3R$-1 is in a low-affinity conformation (Fig. 3A), it can be concluded that the high-affinity site immunoprecipitated from cerebellar or liver extracts is the $InsP_3R$-2. The co-immunoprecipitated low-affinity $InsP_3R$-1 could not be detected by homologous competition experiments because it is not abundant enough [30].

We found here that the binding properties of the $InsP_3R$-2 are different whether it is within the membrane or solubilized and immunoprecipitated. Furthermore, several detergents and chlorpromazine increased the affinity of the $InsP_3R$-2, suggesting that the involvement of hydrophobic interactions is part of the mechanism regulating the receptor. Such regulation can be of pharmacological relevance. If low concentrations of detergent can increase the affinity of $InsP_3R$-2, it is possible that other lipid interacting agents may also affect the properties of the $InsP_3R$. This opens new possibilities in the search for regulators of $InsP_3$-induced Ca^{2+} release. The immunoprecipitation of the cerebellar $InsP_3R$-1 has no effect on its binding affinity. This confirms previous comparisons of the properties of the purified and the membranous cerebellar receptor [11, 39, 40]. Data presented in this paper do not allow the characterization of the regulator of the $InsP_3R$-2. It is unlikely that the shift in affinity was triggered directly by interaction with the detergent, because the effect was not reversed by washing Triton X-100 or chlorpromazine (unpublished observation). More likely, the detergent removed a membrane component in hydrohobic interaction with the receptor. Removing this component induced a 10-fold increase in affinity of the receptor. It was reported previously that an N-terminal fragment of the $InsP_3R$-1 interacts with the membrane fraction [41]. It is possible that such an interaction affects the binding properties of $InsP_3R$-2 and that the regulatory component may be a portion of the receptor. In some cell lines, it has been found that $InsP_3R$-2 has a higher affinity than the $InsP_3R$-1 [14–16]. This means that the regulatory mechanism which maintains the $InsP_3R$-2 in a low-affinity conformation when in the membrane could be absent from some cell types. This could explain the contradictory results reported on the rank order of $InsP_3$ affinity of the different $InsP_3R$ types and particularly on the type 1 and type 2 receptors reported in the literature [11, 14, 17].

The inversion of potency upon solubilization and immunoprecipitation corresponds to the K_d of the liver receptor for the inositol phosphates being decreased 10-fold, whereas the affinity of the cerebellar $InsP_3R$-1 is not modified. We previously found that the Ca^{2+} regulation of $InsP_3$ binding to liver membranes was dependent on the presence of a membrane-associated protein, whereas the inhibition of $InsP_3$ binding to the cerebellar receptor occurs via a direct interaction with the receptor [26]. Interestingly, the $InsP_3$ and Ca^{2+} regulation of the $InsP_3R$-1 mostly depend on interactions with the receptor itself, whereas the regulations of the $InsP_3R$-2 require the presence of accessory components. These differences were also suggested by a study on the regulations of the single channel functions of the purified type 1 and type 2 receptors reconstituted into proteoliposomes. It has been shown that the $InsP_3R$-2 channel had a higher $InsP_3$ affinity than the $InsP_3R$-1 and that the type 2 channel lacks the Ca^{2+} inactivation mechanism that turns off the type 1 channel at micromolar Ca^{2+} concentrations [15]. These experiments essentially confirm our findings on the difference in the binding properties and regulations of the 2 receptor types. However, we have demonstrated here that these differences are mainly due to the extraction of the $InsP_3R$-2 from its

natural membrane environment and that the purified receptor characteristics do not fit with those found under physiological conditions (present work and [26]).

The data we report clearly demonstrate that the apparent affinity of the liver $InsP_3R$-2 for $InsP_3$ is sensitive to its membrane environment. The affinity of the cerebellar $InsP_3R$-1 does not change upon purification, suggesting that this receptor type is not sensitive to its membrane environment. Our data indicate that important factors determining $InsP_3$ receptor properties are their intracellular location and their membrane environment.

This work was carried out in the framework of the European Community TMR network program (fellowship to J. I.).

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