The properties of a subtype of the inositol 1,4,5-trisphosphate receptor resulting from alternative splicing of the mRNA in the ligand-binding domain

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Subtypes of the type-1 inositol 1,4,5-trisphosphate (InsP_3) receptor differ at the mRNA level in two small variably spliced segments. One segment (SI) encodes for a sequence within the InsP_3 -binding domain, thus its presence or absence could affect the functions of the receptor. We have used anti-peptide antibodies to confirm the existence of different subtypes of the InsP_3 receptor (InsP_3R) protein. The antibody against residues 322–332, within the SI region, recognized a 260 kDa polypeptide in membranes prepared from rat cerebellum or cerebral cortex. The cerebellum contained a few percent of the InsP_3R protein having the SI region, whereas the cerebral cortex contained a high proportion of receptors with the SI region. These two tissues were representative of both isoforms, SI^- or SI^+ , and displayed the same [^3H] InsP_3 -binding characteristics. Thus, the

SI region was not involved in the basic properties of the receptor. Deletion of the peptide 316–352 containing the SI segment greatly reduced InsP_3 binding [Miyawaki, Furuichi, Ryou, Yoshikawa, Nakagawa, Saitoh and Mikoshiba (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 4911–4915]. The antibodies against the SI region or against residues 337–349 did not modify the binding of [^3H] InsP_3 in the cortical membranes rich in the SI^+ isoform or in cerebellar membranes. These results suggested that the SI region was not part of the binding site. The subcellular distribution of these two isoforms was then investigated in rat liver. The two isoforms were identified in different membrane fractions and they followed the same subcellular distribution. We suggest that the domain with the SI region may be involved in a function other than InsP_3 -induced Ca^{2+} release.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP_3) is released under hormonal stimulation from phosphatidylinositol 4,5-bisphosphate present in the plasma membrane of the target cells and mediates the release of Ca^{2+} accumulated in an intracellular compartment [1]. Specific InsP_3 receptors (InsP_3Rs) were first identified in the bovine adrenal cortex [2] and now in a number of other tissues [3]. The properties of the InsP_3 -binding site varied from one tissue to another and the K_d of the ligand ranged from 1 nM to 100 nM [3]. This led to the hypothesis that several subtypes of the receptor exist in different tissues [4]. This was confirmed by the identification of different types of receptors synthesized from different genes [5–8]. That different types of receptors were to be found in the same cell type [9,10] suggested that the InsP_3R may be involved in different cell functions and participate in the complex Ca^{2+} release response observed in stimulated cells [11]. The diversity of the InsP_3R was reinforced by the characterization of subtypes of the receptor arising from alternative splicing of two mRNA segments, SI and SII, in the binding domains and in the regulatory domain of the type-1 receptor, respectively [12–14].

However, little is known of the properties and the regulation of the different types of InsP_3R . Preliminary results indicated that the expression of the ligand-binding domains of the different types of the receptors in COS cells had different affinities for InsP_3 [10]. Also, the alternative mRNA splicing events may result in different protein interactions with the ligand. SI, corresponding to the amino acids 318–332, was found only for the type-1 receptor and was localized within the ligand-binding domain, suggesting that its presence or absence can affect InsP_3 binding. Furthermore, mutant proteins with deletion of the amino acids 316–352, a region of the receptor including SI, were expressed in

NG108-15 cells and showed greatly reduced InsP_3 binding [15]. Thus, the SI region can have effects on the affinity of InsP_3 and participate in the complex binding kinetics observed in different tissues. The proportions of the different types and subtypes of the InsP_3R have been determined by PCR amplification of cDNAs derived from mRNAs of various tissues. Thus, the proportion of receptors with segment SI varied during the development of the mouse cerebellum, and also from one peripheral tissue to another [13].

By using antibodies directed against the SI region, we confirm that the proportion of $\text{InsP}_3\text{RSI}^+$ determined by PCR represents the actual proportion of the receptor protein in different tissues. We compared the binding properties of the InsP_3R in cerebellar membranes and in membranes from the cerebral cortex which displayed different proportions of $\text{InsP}_3\text{RSI}^+$.

MATERIALS AND METHODS

Materials

[^3H] InsP_3 (17–21 Ci/mmol) was obtained from Du Pont New England Nuclear, InsP_3 from Calbiochem, and all other reagents from Sigma or Boehringer Mannheim.

Antibodies

Synthetic peptides such as GHPPHMNVNPQQPA corresponding to the 14 C-terminal residues (2737–2750), FEEECLEFQPS corresponding to the residues 322–332 within the SI region, and QDASRSRLRNAQE corresponding to the residues 337–349 of the mouse and rat type-1 InsP_3R [7] were prepared by Neosystem (Strasbourg). The N-terminal amino acid was cross-linked to

ovalbumin by glutaraldehyde. Polyclonal antibodies against the peptides were produced in rabbit and purified on a matrix containing the peptide coupled to Affigel 15 (Bio-Rad) as described previously [16]. Purified antibodies were kept at 1 mg/ml in PBS containing 40 % (v/v) glycerol at -20°C .

Preparation of membrane fractions

Rat cerebella and cortical cortex were homogenized with an ultraturrax blender 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 PMSF and 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 μM pepstatin, 2 μM benzamide, 5 $\mu\text{g}/\text{ml}$ aprotinin, 50 $\mu\text{g}/\text{ml}$ trypsin inhibitor and 1 $\mu\text{g}/\text{ml}$ orthophenanthroline, as described previously [17]. The homogenates were centrifuged for 5 min at 1500 *g*, and the resulting supernatants were centrifuged for 30 min at 50000 *g*. The pellets were washed and resuspended in the washing medium consisting of 250 mM sucrose, 25 mM Hepes/KOH, pH 7.4, supplemented with 1 mM dithiothreitol and the protease inhibitors cocktail. The microsomes were resuspended at 5 mg/ml in the washing medium.

Livers from female Wistar rats (200–250 g) were homogenized with a Dounce homogenizer in the ice-cold homogenization medium, as described previously [17]. The homogenate was centrifuged for 10 min at 1500 *g*, yielding the low-speed pellet. The plasma membrane fraction (PM) was prepared by Percoll gradient centrifugation of the low-speed pellet as described by Prpic *et al.* [18]. The low-speed supernatant was centrifuged for 10 min at 8000 *g* and the microsomal fraction was obtained by centrifuging the 8000 *g* supernatant for 60 min at 100000 *g*. The fractions were washed and resuspended in the following ice-cold washing medium.

Immunoprecipitation of the solubilized InsP_3R

The membrane fractions were washed, resuspended at 5 mg/ml in the washing medium and were incubated for 30 min on ice in the presence of 1 % (w/v) Triton X-100. The mixture was centrifuged for 1 h at 20000 *g*. The supernatant was dialysed against the washing medium containing 0.1 % Triton X-100.

The solubilized proteins were incubated overnight at 4°C either in the presence of 3 to 7 $\mu\text{g}/\text{ml}$ of the affinity-purified antibodies directed against the C-terminal end of the type-1 InsP_3R or directed against residues 322–332 or directed against residues 337–349 or in the presence of 3 to 7 $\mu\text{g}/\text{ml}$ non-specific immunoglobulin G. Samples (1 ml) were 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 washing 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 electrophoresis sample buffer [19] and analysed by Western blotting.

In some experiments the membrane proteins were treated in denaturing conditions as described by Monkawa *et al.* [20]. The solubilized proteins were boiled for 5 min in the washing medium containing 1 % Triton X-100, 6 M urea, 1 % SDS, 20 mM dithiothreitol and the protease inhibitors cocktail. The solubilized proteins were dialysed overnight at 4°C against the washing medium supplemented with 1 mM dithiothreitol and 0.1 % Triton X-100. The concentration of SDS was decreased by a 3-fold dilution of the sample against this medium. The protocol used for immunoprecipitation was the same as for proteins solubilized in non-denaturing conditions.

Equilibrium [^3H] InsP_3 -binding studies on membranes

When indicated, the membranes were preincubated at 4°C in the presence of 30 $\mu\text{g}/\text{ml}$ of the antibodies in the membrane-washing medium. [^3H] InsP_3 binding was measured on ice by adding 100 μl of the membrane fraction to 900 μl of the cytosol-like incubation 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 and supplemented with $(0.5\text{--}1.0) \times 10^4$ c.p.m. of [^3H] InsP_3 as described previously [21]. Non-specific binding was determined in the presence of 5 μM InsP_3 . After a 5 min incubation period, 0.8 ml of the sample was layered on to 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 adjusted to pH 8.3, to minimize the dissociation of the ligand–receptor complex [22]. Radioactivity retained on the filters was counted by scintillation spectrometry.

Equilibrium [^3H] InsP_3 -binding studies on the immunoprecipitated cerebellar InsP_3R

Immunoprecipitated cerebellar material retained to the Sepharose beads was incubated on ice in 500 μl of incubation medium in the presence of [^3H] InsP_3 (1 nM). Non-specific binding was measured in the presence of 5 μM of non-labelled InsP_3 . After a 5 min incubation period, 0.4 ml of the sample was layered on to a Whatman GF/C glass-fibre filter and washed as indicated above.

SDS/PAGE and Western blotting

The membrane fractions were resuspended in SDS sample buffer under reducing conditions as described by Laemmli [19]. SDS/PAGE was performed on 4–10 % acrylamide gradient gels, and the separated proteins were electrotransferred, as described by Towbin *et al.* [23], 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). The intensity of the ECL signal was quantified by densitometry. The picture was analysed with the 'Lecphor' software (Biocom, France).

RESULTS AND DISCUSSION

Characterization of the antibodies

Three different antibodies specific for the type-1 InsP_3R were used in this study. The first antibody was obtained against the 14 C-terminal residues of the type-1 receptor and was previously shown to specifically recognize a 260 kDa protein in cerebellar and in liver membranes [16]. The second antibody was obtained against the residues 322–332 of the mouse and rat type-1 receptor which correspond to a fragment within the SI region. The third antibody was obtained against residues 337–349 which are found in every subtype of the type-1 receptor. This sequence is near the SI region and is assumed to display the same accessibility for the antibodies. The two latter antibodies are directed against fragments which are within the region corresponding to the residues

Figure 1 Immunodetection of the type-1 InsP_3R from cortical and cerebellar membranes with antiserum raised against the 14 C-terminal amino acids of the type-1 InsP_3R (A) or against residues 337–349 (B) or 322–332 (C) of the type-1 InsP_3R

Increasing amounts of proteins from cerebellar or cortical membranes were submitted to SDS/PAGE (4–10% gradient) and then electrotransferred to a nitrocellulose membrane. Size marker positions (in kDa) are indicated to the right. (A) The blot was incubated with antiserum raised against the 14 C-terminal amino acid residues of the type-1 InsP_3R (dilution 1/3000) and with secondary antibodies. (B) The blot was incubated with 15 $\mu\text{g}/\text{ml}$ of affinity-purified antibodies raised against residues 337–349 of the type-1 InsP_3R . (C) The blot was incubated with 9 $\mu\text{g}/\text{ml}$ of affinity-purified antibodies raised against residues 322–332 of the type-1 InsP_3R .

316–352 necessary for InsP_3 binding to the type-1 receptor [15]. The three affinity-purified antibodies were characterized on crude membrane preparations from rat cerebella or rat cerebral cortex. These tissues were chosen because they displayed very different proportions of mRNA coding for the SI region, 15% and 88% in the cerebellum and the cerebral cortex, respectively. By Western blot analysis of the cerebellar and cortical membranes, the three antibodies recognized a protein of 260 kDa (Figures 1A, 1B and 1C). The antibodies against the residues 322–332 and

Figure 2 Western blotting analysis of the solubilized cerebellar InsP_3R immunoprecipitated with affinity-purified antibodies raised against the 14 C-terminal amino acids of the type-1 InsP_3R

The cerebellar membranes were solubilized, then incubated with affinity-purified antibodies raised against the 14 C-terminal amino acids of the type-1 InsP_3R (lanes b, d and e) or with non-specific immunoglobulins (lane c) and pelleted with Sepharose–Protein A beads. Equal amounts of immunoprecipitated material were submitted to SDS/PAGE (4–10% gradient) and then electrotransferred to a nitrocellulose membrane. The blot was incubated either with antiserum raised against the 14 C-terminal amino acids of the type-1 InsP_3R (dilution 1/3000, lanes a, b and c) or with 9 $\mu\text{g}/\text{ml}$ of affinity-purified antibodies raised against residues 322–332 (lane d) or 15 $\mu\text{g}/\text{ml}$ of affinity-purified antibodies raised against residues 337–349 (lane e). Lane a: 20 μg of crude cerebellar membranes were loaded as control. Size marker positions (in kDa) are indicated on the right.

337–349 recognized also several bands of lower molecular mass (Figures 1B and 1C). We assumed that these bands were not related to the InsP_3R because most of these bands were recognized by a single antibody whereas the type-1 receptor should be recognized by the three antibodies. A 100–110 kDa polypeptide was revealed by both antibodies against peptides 322–332 and 337–349. This peptide could result from proteolysis of the receptor, lacking the C-terminal part of the receptor and containing the 1000 N-terminal residues. However, we had added a protease inhibitors cocktail at the different steps of membrane preparation and this should have prevented the proteolysis of the receptor. Furthermore, such proteolysis should have yielded one or several peptides having molecular masses lower than 150 kDa corresponding to the C-terminal part of the receptor. Such peptides were not detected by the antibody against the C-terminal end of the receptor (Figure 1A). Thus proteolysis probably did not occur during the membrane preparation and the Western blotting. Therefore, we assumed that only the band at 260 kDa corresponded to the InsP_3R .

The antibodies against the C-terminal end of the receptor immunoprecipitated a 260 kDa protein from the solubilized cerebellar proteins (Figure 2, lane b). This protein was also recognized in a Western blot by the antibodies against the peptides 322–332 (lane d) and 337–349 (lane e). Clearly, these antibodies bound to the type-1 InsP_3R . As shown in Table 1, the three antibodies immunoprecipitated a protein from solubilized cerebellar membranes which in each case specifically bound [^3H] InsP_3 , indicating that the 260 kDa protein recognized by the antibodies is an InsP_3 -binding protein.

Relative proportion of $\text{InsP}_3\text{RSI}^+$

The signals detected by the different antibodies and amplified by the ECL method were quantified by densitometry. The intensities of the signals revealed by the antibodies against the C-terminal peptide, the peptide 337–349 and the peptide 322–332, respectively, are shown as a function of the amount of cerebellar or

Table 1 Measurement of [^3H]InsP $_3$ binding to the material immunoprecipitated with different antibodies directed against the type-1 InsP $_3$ R

The InsP $_3$ receptors were immunoprecipitated from Triton X-100-solubilized cerebellar membranes with affinity-purified antibodies directed against the C-terminal extremity or against residues 322–332 or 337–349 of the type-1 InsP $_3$ R or with non-specific immunoglobulins G. The immunoreactive material was used for [^3H]InsP $_3$ binding studies as indicated in the Materials and methods section. Total binding was determined in the presence of 13 000 c.p.m. of [^3H]InsP $_3$ (1 nM). Non-specific binding was measured in the presence of 5 μM of non-labelled InsP $_3$. Data are means $\pm$ S.E.M. of triplicate determinations from one representative experiment.

Immunoprecipitation of the InsP $_3$ R with:	Total [^3H]InsP $_3$ binding (c.p.m.)	Non-specific [^3H]InsP $_3$ binding (c.p.m.)
Non-specific IgG	78 $\pm$ 7	68 $\pm$ 7
Antibody against the C-terminal end	2437 $\pm$ 81	94 $\pm$ 9
Antibody against residues 322–332	1002 $\pm$ 7	80 $\pm$ 8
Antibody against residues 337–349	738 $\pm$ 21	90 $\pm$ 2

cortical membranes loaded on the lane (Figures 3A, 3B and 3C). The amplitude of the signal was proportional to the amount of proteins loaded on each lane, at least within the investigated range. Thus, the binding of the primary antibody, the binding of the secondary antibody and the chemiluminescence reaction were not limiting. Therefore, the slope of the intensity of the signal plotted as a function of the amount of proteins loaded on the lanes was directly related to the density of the protein revealed by the antibodies. If we assume that the proportion of proteins transferred from the gel to the nitrocellulose sheet is constant along the gel, the density of the detected antigens in different fractions can be compared provided that they were quantified from the same nitrocellulose membrane. The relative density of the InsP $_3$ R in cerebellar and cortical membranes, revealed by the antibodies against the C-terminal peptide of the InsP $_3$ R, was determined from the slope of the intensity of the signal as a function of the amount of proteins (Figure 3A). The density of the type-1 InsP $_3$ R in the cerebellar membranes was 6-fold higher than in the cortical membranes. These densities were confirmed by using another antibody against the peptide 337–349 which was also specific for the type-1 receptor and was near the SI fragment. The density of the receptors recognized by this antibody was 8-fold higher in the cerebellar membranes than in the cortical membranes (Figure 3B). This confirmed that the density of the type-1 receptor (with or without the SI region) was 6–8-fold higher in cerebellar as compared with cortical membranes.

We then used the antibody against the peptide 322–332, corresponding to a fragment within the SI region, to compare the density of the InsP $_3$ RSI $^+$ in the cerebellum and in the cerebral cortex. Therefore, this antibody should reveal the type-1 receptors containing the SI region. The intensity of the signal was plotted as a function of the amount of proteins loaded on the gel (Figure 3C). The slopes determined for cerebellar and cortical membranes were very similar. This indicated that the density of InsP $_3$ RSI $^+$ was identical in the two tissues.

Thus, there were seven times more type-1 receptor in the cerebellum than in the cerebral cortex, and the same density of InsP $_3$ RSI $^+$ was found in the two tissues. The percentage of InsP $_3$ RSI $^+$ can vary from a few percent to 100% in cortical membranes our results only imply that in cerebellar membranes it will be below 14%. Nakagawa et al. [13] found by PCR experiments that there were 15% and 88% of mRNA coding for InsP $_3$ RSI $^+$ in the cerebellum and in the cerebral cortex, re-

Figure 3 Quantification by densitometry and graphical representation of the amount of antigens detected at 260 kDa by different antibodies

The intensity of the signal expressed in grey levels (arbitrary units) revealed by the antiserum raised against the 14 C-terminal amino acid residues of the type-1 InsP $_3$ R (A) or by affinity-purified antibodies raised against residues 337–349 (B) or raised against residues 322–332 (C) of the type-1 InsP $_3$ R, was expressed as a function of the amount of cerebellar or cortical membrane proteins loaded on the lane.

spectively. To confirm these data, we tried to quantitatively titrate the InsP $_3$ RSI $^+$ in the cortical membranes by an immunoprecipitation approach. The antibodies against the residues 322–332 can be used to immunoprecipitate the native InsP $_3$ RSI $^+$

Figure 4 Immunoprecipitation of InsP_3R solubilized from cortical (A) or cerebellar (B) membranes in denaturing conditions

The solubilized proteins were treated in denaturing conditions as described in the Materials and methods section and were subjected to immunoprecipitation with antibodies against the residues 322–332 (lanes 2 and 4) or antibodies against the C-terminal end of the receptor (lanes 3 and 5). The solubilized proteins treated in denaturing conditions (lane 1), the supernatant of the immunoprecipitation (lanes 2 and 3) and the immunoprecipitated proteins (lanes 4 and 5) were analysed by Western blotting. Samples (15 μg) of the proteins were loaded in lanes 1. The volume of the samples was the same for the solubilized proteins, the supernatants and the immunoprecipitated proteins, in order to compare the amount of precipitated and non-precipitated receptors. The blots were revealed with antibodies against the C-terminal end of the InsP_3R .

from the solubilized cortical membrane proteins. This should result in a decrease of the InsP_3R content in the supernatant as revealed by the antibodies against the C-terminal end of the receptor. However, if we consider that the native InsP_3R can be made up of different subunits [20,24], all the tetramers having at least one SI^+ subunit will be immunoprecipitated and this will lead to an overestimation of the proportion of SI^+ subunits. Therefore, we have denatured the InsP_3R according to the protocol described by Monkawa et al. [20] in order to immunoprecipitate only the subunits of the SI^+ phenotype. We first verified that the receptor denatured in this way migrated at the same molecular mass as the receptor solubilized in the Laemmli sample buffer, confirming that the InsP_3R was totally dissociated into subunits (results not shown). The receptors treated in denaturing conditions were immunoprecipitated with the antibodies against the residues 322–332 or the antibodies against the C-terminal end of the receptor. Figure 4(A) shows that the two antibodies immunoprecipitated the same amount of receptor from the solubilized cortical proteins (lanes 4 and 5) and the receptor was no longer detectable in the supernatant (lanes 2 and 3). Therefore, the antibodies against the SI domain recognized most of the type-1 receptor, indicating that in the cortical membranes the InsP_3R is mostly of the SI^+ phenotype. The same experiment has been performed with cerebellar receptor treated in denaturing conditions. The antibodies against the SI domain immunoprecipitated much less InsP_3R than the antibodies against the C-terminal end of the receptor (Figure 4B, lanes 4 and 5 respectively) and indeed the supernatant was depleted of InsP_3R by the anti-(type-1 receptor) antibodies but no depletion was apparent with the anti-(SI domain) antibodies (Figure 4B, lanes 3 and 2 respectively). This confirms that the $\text{InsP}_3\text{RSI}^+$ is present in low proportion in cerebellar membranes. These results are in agreement with the data obtained from the mRNA detection, indicating that the SI^+

Figure 5 Inhibition of $[^3\text{H}]\text{InsP}_3$ binding to cerebellum and cerebral cortex membranes by increasing concentrations of InsP_3

The cerebellar membranes (0.9 mg) or the cortical membranes (3.8 mg) were incubated for 5 min at 4 $^{\circ}\text{C}$ in 1 ml of incubation medium in the presence of 13 000 c.p.m. of $[^3\text{H}]\text{InsP}_3$ (0.5 nM) and increasing concentrations of non-labelled InsP_3 (1 nM–5 μM). Specific bindings in the absence of inhibitor were 720 c.p.m. and 330 c.p.m. with the cerebellar and the cortical membranes respectively. Data are means of triplicate determinations of one representative experiment.

subunits are present in high proportion in cortical membranes and in low proportion in cerebellar membranes.

Influence of the SI region on the binding of InsP_3

Since the cerebellum contains mainly $\text{InsP}_3\text{RSI}^-$ and the cerebral cortex $\text{InsP}_3\text{RSI}^+$, these two tissues could be used to investigate the effect of the SI region on the InsP_3 -binding properties under native conditions. A competition binding experiment was performed on the same cerebellar and cortical membrane preparations as those used in the experiment shown on Figure 1. Both binding data were well described by a single-site model, suggesting that the two InsP_3R subtypes which co-exist in each membrane preparation displayed the same affinity for the ligand. In agreement, the K_d values determined from this experiment were 28 nM and 23 nM and the B_{max} values were 17 and 2 pmol/mg respectively (Figure 5). The binding capacity of the cerebellar membranes was 8-fold higher than the cortical membranes. This was the same ratio as that found for the receptor protein detected on the Western blot (Figures 3A and 3B). Previously the type-1 InsP_3R was found to be in the majority in these two tissues [9,25]. Therefore, we concluded that both the cerebellar type and the cortical type of the receptors could bind InsP_3 and display the same affinity for InsP_3 . Newton et al. [10] found that expression of the ligand-binding domains of the type-1 receptor containing or lacking the SI fragment had the same affinity for InsP_3 . We extended this finding to the entire receptor in its natural environment. Joseph and Rice [26] found that the affinity of $[^3\text{H}]\text{InsP}_3$ for the cerebral cortex membranes was higher than for cerebellar membranes. This difference with our present results could be due to the different pH at which the binding experiments were done. It was also reported that InsP_3 displayed a Ca^{2+} -releasing activity from cortical microsomes having $\text{InsP}_3\text{RSI}^+$ and from cerebellar microsomes having $\text{InsP}_3\text{RSI}^-$ [26]. This suggests that the two subtypes of the receptor had the same affinity for InsP_3 and the same Ca^{2+} -releasing activity.

The InsP_3R function can be regulated in different ways including phosphorylation by kinases, allosteric regulation by

ATP and feedback regulation by cytosolic Ca^{2+} [27]. The site for the phosphorylation by the cyclic AMP-dependent protein kinase and the sites for ATP binding have been localized in the regulatory domain of the receptor, at a distance more than 1000 residues from the SI regions (see [28]). Thus the SI region may not influence regulation by phosphorylation or by ATP binding. Because the sites involved in the regulation by Ca^{2+} remain unknown, we wondered whether the SI region could influence the Ca^{2+} effect. It was reported that Ca^{2+} inhibited $[^3\text{H}]\text{InsP}_3$ binding to cerebellar membranes [29] and this effect was due to an interaction with the protein calmodulin [30]. In agreement, we observed this effect even when the incubation medium was continuously changed by perfusion. Thus it was at least partly due to direct action of Ca^{2+} on the receptor or on the calmodulin and was not secondary to the production of an intermediary metabolite [22]. Subsequently, we investigated the effect of Ca^{2+} on the binding of $[^3\text{H}]\text{InsP}_3$ to cerebellar and cortical membranes. The binding of 0.5 nM of $[^3\text{H}]\text{InsP}_3$ was inhibited by 57% and 54% in the presence of 1 μM Ca^{2+} in cerebellar and cortical membranes respectively (results not shown). Therefore, the $\text{InsP}_3\text{RSI}^+$ was as sensitive to Ca^{2+} as the cerebellar $\text{InsP}_3\text{RSI}^-$, and that SI region was not implicated in the interaction with Ca^{2+} or the putative Ca^{2+} -binding protein calmodulin described by Danoff et al. [30].

Effects of the antibodies on the $[^3\text{H}]\text{InsP}_3$ binding

Because we found no differences in the properties of the $\text{InsP}_3\text{RSI}^+$ and the $\text{InsP}_3\text{RSI}^-$, this suggested that the SI region is not involved in the InsP_3 binding. We then tested the effect of the antibodies against the peptides 322–332 or 337–349 on the $[^3\text{H}]\text{InsP}_3$ binding to cortical and cerebellar membranes. As seen above, the InsP_3R immunoprecipitated with the antibodies against the SI region or against the peptide 337–349 specifically bound $[^3\text{H}]\text{InsP}_3$. This could be an indication that the antibodies did not inhibit the binding of $[^3\text{H}]\text{InsP}_3$. However, considering that the receptor is a tetramer, the occupancy of one of the monomers by the antibody would be enough to precipitate the tetramer. Thus, the binding site of the three free subunits would remain accessible to the ligand. Because both antibodies against the SI region or against peptide 337–349 recognized the native form of the receptor, they can be used in binding experiments on membrane preparations. The cortical membranes, which contain mainly InsP_3R having the SI region, were used to investigate the effect of the antibody against peptide 322–332 on the InsP_3 binding. The cerebellar membranes were used with the antibody against peptide 337–349. The concentration of the antibodies was fixed at 200 nM, which has been found to be saturating in Western blot experiments and in immunoprecipitation experiments, and the concentration of membranes was adjusted to have a concentration of $[^3\text{H}]\text{InsP}_3$ binding sites 5- to 10-fold lower than the antibody concentration to be sure that the four subunits constituting the receptor were occupied. Neither the antibody against the SI region nor the antibodies against peptide 337–349 or the C-terminal peptide inhibited the binding of $[^3\text{H}]\text{InsP}_3$ to the membranes (Table 2). These results suggested that the region between residue 322 and residue 349 was not part of the binding site. The fact that the deletion of the peptide 316–352 greatly reduced InsP_3 binding [15] suggested that this region was necessary to maintain the conformation of the binding site but is not part of it.

Subcellular distribution of $\text{InsP}_3\text{RSI}^+$ and $\text{InsP}_3\text{RSI}^-$

If the SI region has no effect on the binding parameters of InsP_3 to the type-1 receptor or on its regulation by Ca^{2+} , it is possible

Table 2 Effect of the antibodies directed against the C-terminal end and residues 322–332 and 337–349 of the InsP_3R on $[^3\text{H}]\text{InsP}_3$ binding to cortical and cerebellar membranes

The cortical (8 mg/ml) and cerebellar membranes (0.8 mg/ml) were incubated overnight at 4 °C with 30 $\mu\text{g}/\text{ml}$ of the antibodies or the non-specific IgG. Measurements of specific $[^3\text{H}]\text{InsP}_3$ binding were performed as indicated in the Materials and methods section. Data are means of triplicate determinations from one representative experiment. Abbreviation: ND, not determined.

Additions	Specific $[^3\text{H}]\text{InsP}_3$ binding (c.p.m./mg)	
	Cerebellum	Cerebral cortex
Non-specific Ig G	12181	917
Antibodies against the C-terminal end	16517	1205
Antibodies against residues 322–332	ND	1053
Antibodies against residues 337–349	15794	ND

that it is localized in different areas of the cell. The antibodies used in the present work did not recognize a single band in Western blot, consequently they could not be used to directly localize the InsP_3R subtypes by immunohistochemical methods. However, these antibodies were useful to reveal the InsP_3R on a Western blot in different membrane fractions and to study its subcellular distribution. We previously found that the type-1 InsP_3R was not homogeneously distributed in rat liver membrane fractions and it was found at a high density in a fraction enriched in markers of the plasma membrane [16]. It has been found that the liver contains nearly equal amounts of SI^+ and SI^- subunits [13]. We analysed the subcellular distribution of the $\text{InsP}_3\text{RSI}^+$ and $\text{InsP}_3\text{RSI}^-$ by comparing the proportion of the two subtypes of the receptor in two liver membrane fractions. We first wished to validate the method allowing the comparison of the proportion of $\text{InsP}_3\text{RSI}^+$ in two different membrane preparations. The cortical InsP_3R transferred on a single blot was revealed with the antibody against residues 322–332 and the antibody against the C-terminal end of the receptor (Figure 6A) and the slopes of the intensity of the signals as a function of the amounts of proteins were determined. The ratio of the slope for the antibody against the SI region to the slope for the antibody against the C-terminal end was 3.5 ± 0.9 ($n = 3$). For given antibody dilutions, this ratio depended on the proportion of $\text{InsP}_3\text{RSI}^+$. Accordingly, the same antibody dilutions used to reveal the receptor in cerebellar membranes gave a ratio of 1.3 ± 0.1 ($n = 4$). This indicated that the method could be used to compare the proportion of the $\text{InsP}_3\text{RSI}^+$ in different membrane fractions.

The analysis of the liver membrane fractions by Western blotting with the antibody against the SI region gave a very weak signal. We then enriched the preparation in InsP_3R by immunoprecipitating the type-1 receptor with the antibody against the C-terminal peptide of the receptor from different solubilized liver membrane fractions. Increasing amounts of the immunoprecipitated receptors were analysed by Western blotting and revealed with antibodies against the SI region or against the C-terminal peptide of the type-1 receptor. Both antibodies gave signals at 260 kDa which were quantified by densitometry. The intensities of the signal increased linearly with the amount of proteins loaded on the lane (Figure 7). The ratio of the slopes was determined to be 0.8 ± 0.2 ($n = 3$) in the liver PM enriched in InsP_3R . We also prepared, by subcellular fractionation, a liver microsomal fraction enriched in markers of the endoplasmic reticulum, SERCA2b [sarco(endo)plasmic reticulum Ca^{2+} pump] [16] and in calreticulin [31]. Similarly, we determined in this

Figure 6 Comparison of the proportion of $\text{InsP}_3\text{RSI}^+$ in cortical and cerebellar membranes

Increasing amounts of proteins from cortical (**A**) or cerebellar (**B**) membranes were submitted to SDS/PAGE (4–10% gradient) and then electrotransferred to a nitrocellulose membrane. Size marker positions (in kDa) are indicated to the right. (**A** and **B**): The blots from the same gel transferred to a nitrocellulose membrane were incubated with 9 $\mu\text{g}/\text{ml}$ of affinity-purified antibodies raised against the residues 322–332 (α) and with antiserum raised against the 14 C-terminal amino acids residues of the type-1 InsP_3R (dilution 1/3000) (β) and then with secondary antibodies.

fraction a ratio of the two slopes of 1.0 ± 0.3 ($n = 3$). This was quite similar to that found with the receptor in the PM. Clearly the proportion of $\text{InsP}_3\text{RSI}^+$ did not differ from one membrane fraction to another, suggesting that the two receptor subunits were distributed in the same way. This homogeneous distribution of two subunits of the InsP_3R is consistent with the recent finding that different receptor subunits may assemble to form heterotetramers [20,24].

Conclusion

We characterized the presence of different isoforms of the type-1 InsP_3R resulting from an alternative splicing of the mRNA. Previously, the existence of these isoforms was suspected only from PCR experiments. We confirmed that the cerebellum contained a low percentage of the InsP_3R protein having the SI region, whereas the cerebral cortex contained a high proportion of receptors with the SI region. These two tissues can be used to study the properties of each isoform of the receptor. We found that the SI region was not involved in the basic properties of the receptor since both forms (cortical SI^+ or cerebellar SI^-) displayed the same binding parameters. These two isoforms have been identified in liver membranes where they followed the same subcellular distribution. Thus either the SI region has no influence on the InsP_3R properties or it is involved in functions other than the ligand binding. It is not likely that SI plays some role in the Ca^{2+} channel function because it is far from the transmembrane domains of the receptor near the C-terminal end of the protein (see [32]). Possibly, SI plays some role in the interaction of the

Figure 7 Determination of the proportion of $\text{InsP}_3\text{RSI}^+$ in liver PM

Upper panel: The proteins of the liver PM enriched in InsP_3R were solubilized, then incubated with affinity-purified antibodies raised against the 14 C-terminal amino acids of the type-1 InsP_3R and pelleted with Sepharose–Protein A beads. The immunoprecipitated proteins were extracted and submitted to SDS/PAGE (4–10% gradient) and then electrotransferred to a nitrocellulose membrane. Each lane was loaded with an increasing volume (10–30 μl) of immunoprecipitated proteins and incubated either with antiserum raised against the 14 C-terminal amino acids of the type-1 InsP_3R (dilution 1/3000) (α) or with 9 $\mu\text{g}/\text{ml}$ of affinity-purified antibodies raised against the type-1 $\text{InsP}_3\text{RSI}^+$ (residues 322–332) (β). Size marker positions (in kDa) are indicated to the right. Lower panel: The filled squares are related to the signal detected by antiserum raised against the 14 C-terminal amino acids of the type-1 InsP_3R and the open circles to the signal detected by the affinity-purified antibodies raised against the type-1 $\text{InsP}_3\text{RSI}^+$.

InsP_3R with other proteins in the cell and participates in the organization of the intracellular Ca^{2+} stores. Such interactions have been described in the Purkinje cells of the cerebellum where the type-1 InsP_3R participated in the formation of stacks of endoplasmic reticulum [33,34] and in other tissues it interacted with cytoskeletal proteins like ankyrin [35,36] or actin [37].

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