Detergent Solubility of the Inositol Trisphosphate Receptor in Rat Brain Membranes

EVIDENCE FOR ASSOCIATION OF THE RECEPTOR WITH ANKYRIN*

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Rat cerebellar membranes sedimenting at 10,000 × g contain significantly greater amounts of [3H]d-myo-inositol 1,4,5-trisphosphate (IP3) binding sites that are resistant to extraction by Triton X-100 than membranes sedimenting at 100,000 × g. Scatchard analysis of the Triton X-100-resistant binding site revealed the presence of a single binding site with an 8-fold higher affinity for IP3 than present in 10,000 × g membranes. The Triton X-100-resistant binding site displayed high specificity for Ins(1,4,5)P3 and was sensitive to inhibition by both heparin and calcium. A polyclonal antibody to the C terminus of the IP3 receptor (IP3R) and bioiodylated concanavalin A recognized the same 235 kDa band in Western blots of membrane and Triton X-100-insoluble fractions. The ligand binding activity of the IP3R could be measured in immunoprecipitates obtained from detergent-soluble extracts treated with IP3R antibody. An antibody to chick erythrocyte ankyrin also immunoprecipitated [3H]IP3-binding sites and immunoreactive IP3R protein. These effects of ankyrin antibody were prevented by preadsorption to erythrocyte ghosts and were not reproduced by specific antibodies. Cross-reactivity of the ankyrin antibody with the non-ionic detergent Triton X-100, and the detergent binding of this molecule to an oligomeric receptor protein when IP3R antibody immunoprecipitates were boiled in SDS buffers or when membranes were alkali-treated. These results suggest that a population of IP3R in brain may be attached to an isoform of ankyrin. This may mediate interactions of the IP3R with the cytoskeleton and account for observations regarding the detergent insolubility of the protein.

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1 The abbreviations used are: IP3, d-myo-inositol 1,4,5-trisphosphate; IP3R, IP3 receptor; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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pylarnes prepared from heparinized blood as described previously (26). was measured using a polyethyleneglycol precipitation assay. Aliquots centrifuged at 100,000 g for 45 min. Rat erythrocyte ghosts were prepared by heparinized blood as described previously (26). The Triton X-100-insoluble fraction with 1 M KCl, 1% Triton X-100, [3H]IP3 binding was approximately three times greater in extracts prepared from microsomal membranes. This difference in Triton extraction of IP3-binding sites was also observed in measurements of [3H]IP3 binding to the detergent-solubilized extracts (Fig. 1B). At 1% Triton X-100, [3H]IP3 binding was approximately three times greater in extracts prepared from microsomal membranes. This difference in detergent extractability was observed over a wide range of detergent concentrations (0.1–1.0%), solubilization times (up to 2 h) and was also independent of whether the solubilization buffer contained either 50 mM Tris-HCl, 0.32 M sucrose or 120 mM KCl (data not shown). Treatment of Triton X-100-insoluble membranes with 1 mM KCl, 1% CHAPS, or 60 units/ml DNase I did not solubilize additional [3H]IP3-binding sites (data not shown).

The parameters of IP3 binding to low speed membranes and to the Triton X-100-insoluble residue were measured by determining the amount of [3H]IP3 bound over a wide range of labeled IP3 concentrations (Fig. 2). Scatchard analysis of the data obtained from low speed membranes gave values of 23.3 ± 1.5 nM for the apparent Kd and 15.8 ± 1.9 pmol/mg protein for the Bmax. These numbers are within the published range of values obtained with different preparations of cerebellar membranes and determined using a radiolabel displacement method (summarized in Ref. 27). However, the Triton X-100-resistant binding site displayed an 8-fold higher affinity for IP3 with an apparent Kd of 3.3 ± 0.5 nM and a Bmax of 1.8 ± 0.5 pmol/mg protein. This indicates that the Triton-resistant sites would be expected to represent only 11% of the total binding sites in low speed membranes. A component of binding sites having a low capacity and a high affinity was not readily detectable in binding measurements on low speed membranes (Fig. 2A), and such a component would also not be easy to detect in assays based on displacement of labeled ligand (33).

The IP3R purified from rat cerebellum has been shown to be easy to detect in assays based on displacement of labeled ligand (33).
been shown to be an inhibitor of IP3 binding and this fraction contains tightly bound "calmodin." Heparin has been shown to be an inhibitor of IP3 binding and IP3-mediated solubilization. Our results suggest that Ca2+ can directly inhibit the IP3R in the Triton X-100-insoluble fraction.

Reactivity of Triton-soluble and insoluble Fractions to IP3R Antibody and Biotinylated Concanavalin A—Polyclonal antibodies to the IP3R were used to determine if the Triton X-100-soluble and insoluble IP3R are antigenically related. Fig. 5A shows that an antibody raised against the 18 amino acids at the COOH terminus of the IP3R was capable of specifically immunoprecipitating IP3R from Triton X-100 extracts of low speed membranes as measured by a loss of [3H]IP3 binding activity. More than 90% of the bound [3H]IP3 in a 100 μg of extract protein could be specifically removed by 20 μg of antibody (Fig. 5A). Analysis of the immunoprecipitates on SDS gels followed by Coomasie Blue staining revealed a 235 kDa band that is present in samples treated with immune serum but absent in samples treated with preimmune serum (Fig. 5B). Additional experiments using 10% SDS-polyacrylamide gel electrophoresis and silver staining provided no evidence for the specific communoprecipitation of other proteins together with the IP3R (data not shown). A 235 kDa band was also the major immunoreactive species recognized when the antibody was used to probe Western blots of microsomal, low speed, and Triton X-100-insoluble membranes (Fig. 5C). These results indicate that the receptor protein in detergent-soluble and insoluble fractions are antigenically identical.
IP₃R and the Cytoskeleton

Fig. 2. Saturation binding analysis of IP₃-binding sites in Triton X-100-insoluble residue prepared from low speed membranes. Low speed membranes (A) or the Triton X-100-insoluble material prepared from low speed membranes (B) were incubated (0.5 mg protein/ml) with increasing concentrations of [³H]IP₃ (3,000–700,000 counts/minute in a final volume of 0.35 ml). Binding to both fractions was assayed using a filtration assay. The data shown are expressed as the amount of [³H]IP₃ specifically bound as a function of the total added ligand. For each [³H]IP₃ concentration, nonspecific binding was measured in the presence of 20 µM IP₃. Apparent Kᵣ and Bmax values were determined from Scatchard plots of the data (inset). The different symbols represent data from two independent experiments.

(38) raise the possibility that the IP₃R may interact with cytoskeletal proteins. In principle, the protocol in Table I used with specific cytoskeletal antibodies could permit the detection of such interactions if they were of sufficiently high affinity to survive the detergent extraction conditions used. Table I shows that an antibody raised against chick erythrocyte ankyrin (30, 31) could also immunoprecipitate specific [³H]IP₃-binding sites from detergent extracts of cerebellar microsomal membranes. This activity of the antibody could be completely prevented by preadsorption to rat erythrocyte ghost membranes, a procedure that did not interfere with the ability of IP₃R antibody to immunoprecipitate ligand-binding sites. An antibody to chick α-spectrin was inactive in this assay. Quantitatively similar data were obtained when the experiments shown in Table I were performed using detergent extracts prepared from low speed membranes (data not shown).

Western blotting analysis of ankyrin antibody immune
FIG. 3. Specificity of the Triton X-100-insoluble IP$_3$ receptor. The binding of [H]$^{3}IP_{3}$ (3 nM) to the Triton X-100-insoluble IP$_{3}$ receptor obtained from low speed membranes was measured in the presence of a range of concentrations of unlabeled inositol phosphate analogues. The data are expressed as a ratio of the binding measured in the presence of [H]$^{3}IP_{3}$ alone (Bo). Each data point is the mean of triplicate determinations and is representative of two experiments showing similar results.

FIG. 4. Inhibition by Ca$^{2+}$ and heparin of [H]$^{3}IP_{3}$ binding to the Triton X-100-insoluble receptor. A, binding of [H]$^{3}IP_{3}$ to the Triton X-100-insoluble IP$_{3}$ receptor prepared from low speed membranes was measured using an incubation buffer in which the usual 1 mM EDTA was replaced by 1 mM nitrilotriacetate. Prior to addition of [H]$^{3}IP_{3}$ to this buffer, various concentrations of CaCl$_{2}$ were added to generate a range of free Ca$^{2+}$ concentrations as measured directly with a Ca$^{2+}$-sensitive electrode. The data shown are the mean of duplicate determinations, and the different symbols represent the results of independent experiments. B, the influence of increasing concentrations of porcine heparin on binding of [H]$^{3}IP_{3}$ to the Triton X-100-insoluble binding site was measured using the standard incubation conditions as described under “Experimental Procedures.” The data are from a representative experiment.

complexes with IP$_3$R antibody indicates a dose-dependent immunoprecipitation of IP$_3$R protein (Fig. 6A). The maximal amount of IP$_3$R protein immunoprecipitated by ankyrin was 18% of that immunoprecipitated by 100 µg of IP$_3$R antibody as measured by densitometric analysis of immunoblots (Fig. 6B). Western blotting analysis of IP$_3$R antibody immunoprecipitates with ankyrin antibody are shown in Fig. 7A. Chick erythrocyte ankyrin antibody recognizes several major immune-reactive bands in detergent extracts of brain microsomal membranes, with mobilities of approximately 220, 200, 79, and 65 kDa. The 200-kDa species has approximately the same molecular mass as the ankyrin isofrom found in rat erythrocyte ghosts (Fig. 7A, lane G). The molecular mass of the human brain isofrom of ankyrin on SDS gels is reported to be 220 kDa (39). An immunoreactive species in this molecular weight range is detected by ankyrin antibody in IP$_3$R antibody immunoprecipitates (Fig. 7A, lane I). The bands at 79 and 65 kDa are unidentified but may be proteolytic products of ankyrin. These species were also observed in IP$_3$R immunoprecipitates.

The mobilities of the IP$_3$R and the band recognized by erythrocyte ankyrin antibody in IP$_3$R antibody immunoprecipitates are difficult to distinguish on SDS gels. It is therefore possible that the immunoprecipitation of IP$_3$R protein and [H]$^{3}IP_{3}$-binding sites by ankyrin antibody could be the consequence of cross-reactivity with the IP$_3$R protein itself. Two kinds of control experiments were done to examine this possibility. Davis and Bennett (40) have previously shown that treatment with 0.1 M NaOH for 30 min is required to remove tightly bound ankyrin from brain membranes. This treatment, as expected, had no effect on the IP$_3$R (Fig. 8). However, alkali treatment removed all ankyrin antibody-immunoreactive proteins from the cerebellar membranes including the high molecular weight polypeptide. In Fig. 9 detergent extracts were immunoprecipitated with IP$_3$R antibody, and the immunoprecipitates were boiled in the presence of 2% SDS. The sample was diluted to reduce the SDS concentration and immunoprecipitated again with IP$_3$R antibody. The data show that disruption of protein-protein interactions by boiling in the presence of SDS can prevent the immunoprecipitation of an immune-reactive ankyrin polypeptide (Fig. 9, right panel) without affecting IP$_3$R immunoprecipitation (Fig. 9, left panel).

The data in Figs. 8 and 9 suggests that the ability of
FIG. 5. Immune and concanavalin A reactivity of the IP₃R in different membrane fractions. A, increasing amounts of polyclonal IP₃R antibody were incubated with 100 µg of Triton X-100-solubilized extract prepared from low speed membranes. The extracts were supplemented with 5 µl/ml of protease mixture and was incubated at 4 °C for 2 h with antibody and an additional 2 h with 100 µl of 20% (v/v) protein A-Sepharose beads. After removal of the beads by centrifugation, the supernatants were assayed in triplicate for [³H]IP₃ binding. The data shown are mean ± S.E. of determinations from two separate experiments. The control (100%) value was obtained from extracts incubated without antibody (1.30 ± 0.05 pmol/mg protein). B, the protein A beads recovered after immunoprecipitation of 100 µg of extract was quenched with SDS sample buffer, electrophoresed on a 7% SDS gel, and stained with Coomassie Blue. Conditions were: lane 1, no antibody; lane 2, 100 µg of preimmune antibody; lane 3, 100 µg of immune antibody. A 235-kDa band corresponding to the IP₃R is seen only in lane 3. C, membrane fractions were electrophoresed on a 7% SDS gel and the protein transferred to nitrocellulose. The blots were developed with a 1:500 dilution of antibody and processed as described under “Experimental Procedures.” Lane 1, 10 pg of microsomal membrane; lane 2, 10 µg of low speed membrane; lane 3, 10 µg, or lane 4, 100 µg of Triton X-100-insoluble membrane. D, 100 µg of microsomal membrane (lane 1), low speed membrane (lane 2), or Triton X-100-insoluble membranes (lane 3) were mixed with 50 µl of a buffer containing 100 mM Tris (pH 8.3), 10 mM EDTA, and 2% SDS. The volume was adjusted to 1 ml with brain solubilization buffer, and the samples were immunoprecipitated with 100 µg of IP₃R antibody as described above. In the case of Triton X-100-insoluble membranes the samples were boiled for 3 min after addition of the SDS containing buffer to effect solubilization. The immunoprecipitates were electrophoresed, transferred to nitrocellulose, and probed with biotinylated concanavalin A as described under “Experimental Procedures.” The amount of sample loaded in each lane corresponds to the amount of IP₃R immunoprecipitated from 40 µg of starting protein.

erythrocyte ankyrin antibody to immunoprecipitate [³H]IP₃-binding sites and immunoreactive IP₃R protein is not a reflection of cross-reactivity of the antibody with the IP₃R. The IP₃R antibody used in this study was raised to the COOH-terminal portion of the Type I IP₃R. Fig. 10 shows that preincubation of the antibody with 100 µg/ml of the COOH-terminal peptide completely blocked the ability of the IP₃R antibody to recognize its antigen on Western blots and also blocked the ability of the antibody to immunoprecipitate [³H]IP₃-binding sites from detergent extracts (Fig. 10B). Addition of the COOH-terminal peptide did not affect the recognition properties of erythrocyte ankyrin antibody on Western blots (Fig. 10A). However, the peptide was able to partially inhibit the ability of the antibody to immunoprecipitate [³H]IP₃ binding (Fig. 10B). In three experiments (mean ± S.E.) the inhibitory effect of 100 µg/ml COOH-terminal peptide was 60.8 ± 9.0% with ankyrin antibody and 98.1 ± 2.8% with IP₃R antibody. This suggests that the COOH terminus of the IP₃R may be involved in the interaction with immunoreactive ankyrin. A second ankyrin antibody, raised against human erythrocyte ankyrin, was also tested in this system. This antibody (denoted ankyrin-Ab2) was strongly reactive in Western blots against the 200-kDa erythrocyte-like ankyrin in brain extracts but barely recognized any of the other bands
recognized by ankyrin-Ab1 (Fig. 7B, lanes G and E). Correspondingly, ankyrin-Ab2 failed to immunoprecipitate [3H]IP₃ binding sites or immunoreactive IP₃R protein (data not shown). Traces of the 200-kDa erythrocyte-like ankyrin polypeptide were detected by ankyrin-Ab2 in IP₃R and ankyrin-Ab1 immunoprecipitates (Fig. 7B, lanes I and A).

DISCUSSION

Several studies have described difficulties in solubilizing the IP₃R protein from membranes using 1% Triton X-100 as originally used for the purification of the IP₃R from rat cerebellum. In some tissues, e.g. vas deferens (2), the degree of solubilization could be improved by using higher detergent concentrations or longer incubation times. In other tissues, e.g. adrenal cortex (22) or rat liver (38), substantial amounts of the receptors resist solubilization by Triton X-100. In the brain, as in other tissues, the IP₃R is distributed in several different membranes (for review, see Refs. 41-44). The present study shows that receptors located in different membrane fractions in any given tissue may show differences in Triton X-100 solubility. Rat cerebellum membranes sedimenting at 10,000 x g contained a larger fraction of Triton X-100-insoluble IP₃R than microsomal membranes sedimenting at 100,000 x g. This does not appear to be a reflection of different types of IP₃R in detergent-soluble and insoluble fractions, at least as judged by immunoreactivity to an IP₃R antibody, concanavalin A binding, and sensitivity to inhibition by heparin and calcium. The only difference that can be detected is the substantially higher affinity for IP₃ shown by the Triton X-100 insoluble IP₃R. It is of interest that membranes possessing high affinity IP₃-binding sites (appKᵦ < 5 nM), such as those isolated from the adrenal cortex or rat liver, are particularly resistant to Triton solubilization (22, 38). The low concentrations of [3H]IP₃ routinely used in binding assays favors occupation of the high affinity sites on the Triton X-100-insoluble IP₃R. However, the Bmax values indicates that this site represents only 11% of the total IP₃R present in low speed membranes (Fig. 2). The amount of immunoreactive IP₃R in the rat Triton X-100-insoluble fraction appears higher than expected on the basis of ligand binding measurements (Fig. 5C) suggesting that some of the protein in the detergent-insoluble fraction may be inactive with respect to ligand binding. Compared to the rat, 52% of the ligand-binding sites in low speed membranes of mouse cerebellum were found to be Triton X-100 insoluble (data not shown; see also Ref. 3). This difference is surprising, in view of the almost identical sequences of the mouse and rat Type I IP₃R (6, 7).

Insolubility of membrane proteins in Triton X-100 is often taken as evidence of association of the protein with the cytoskeleton (45, 46). Other possible explanations include aggregation of the protein or a very tight association with phospholipids that are not extracted by Triton X-100 (26). While the present study does not exclude these possibilities, in the case of the IP₃R there is experimental evidence to suggest an interaction of the IP₃R with the cytoskeleton. Rossier et al. (23) have shown that pretreatment of liver homogenates with cytochalasin B alters the distribution of the IP₃R in subcellular fractions. However, incubation of

TABLE I

Measurement of [3H]IP₃ binding to sites immunoprecipitated with antibodies to IP₃R or to cytoskeletal proteins

<table>
<thead>
<tr>
<th>Additions</th>
<th>IP₃ binding (cpm/70-μl beads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antibody</td>
<td>187 ± 10</td>
</tr>
<tr>
<td>Preimmune IP₃R Ab</td>
<td>153 ± 7</td>
</tr>
<tr>
<td>IP₃R antibody</td>
<td>3307 ± 545</td>
</tr>
<tr>
<td>IP₃R antibody + IP₃ (10 μM)</td>
<td>145 ± 13</td>
</tr>
<tr>
<td>IP₃R antibody (preadsorbed)</td>
<td>3145 ± 157</td>
</tr>
<tr>
<td>Ankyrin antibody</td>
<td>1027 ± 110</td>
</tr>
<tr>
<td>Ankyrin antibody + IP₃ (10 μM)</td>
<td>133 ± 17</td>
</tr>
<tr>
<td>Ankyrin antibody (preadsorbed)</td>
<td>288 ± 13</td>
</tr>
<tr>
<td>Spectrin antibody</td>
<td>308 ± 39</td>
</tr>
<tr>
<td>Spectrin antibody + IP₃ (10 μM)</td>
<td>140 ± 4</td>
</tr>
<tr>
<td>Spectrin antibody (preadsorbed)</td>
<td>217 ± 25</td>
</tr>
</tbody>
</table>

[FIG. 6. Precipitation of immunoreactive IP₃R protein by ankyrin antibody. A, solubilized microsomes (20 μg) were immunoprecipitated with increasing amounts of ankyrin antibody (lanes 2-6) or 100 μg of IP₃R antibody (lane 7) and the immunoprecipitates were Western blotted with IP₃R antibody. The immunoblots were processed with an enhanced chemiluminescence assay kit. The amounts of ankyrin antibody added in the lanes were: 1) none, 2) 8.3 μg, 3) 25 μg, 4) 50 μg, 5) 75 μg, 6) 100 μg. B, the intensity of the band corresponding to the IP₃R was quantitated by densitometry and is expressed as a percentage of the IP₃R protein immunoprecipitated by a maximal amount of IP₃R antibody (lane 7).]
tracts of untreated was washed twice in isolation buffer and solubilized with Triton X-100 as described under "Experimental Procedures" with 100 pg of either IP₃R antibody. The immunoprecipitates were either washed and quenched in SDS sample buffer (1) or resuspended and boiled for 5 min in 100 μl of a buffer containing 1% SDS (w/v), 50 mM Tris-HCl, pH 8.3, 5 mM EDTA. After removal of the protein A-Sepharose beads, the supernatants were diluted to 1 ml with solubilization buffer (minus dithiothreitol) and reimmunoprecipitated with IP₃R antibody (2). The immunoprecipitates were probed on Western blots with IP₃R or erythrocyte ankyrin antibody. The amount of protein loaded in each lane was equivalent to that immunoprecipitated from 20 μg of extract for IP₃R antibody and 80 μg of extract for ankyrin antibody.

In the present study, we have shown that an antibody against the COOH terminus of the Type I IP₃R can immunoprecipitate 90% of the [³H]IP₃-binding sites from a detergent extract of cerebellum membranes (Fig. 5A). The cerebellum is believed to contain mRNA encoding two and possibly three distinct IP₃ receptors (8, 10), none of which share the same COOH terminus as the Type I IP₃R. Assuming the antibody used in this study is specific to the Type I IP₃R, the immunoprecipitation data would suggest that other types of IP₃ receptors contribute <10% of the ligand binding and/or are associated with Type I receptors to form heterotetramers. We also show in the present study that it is possible to measure ligand binding to immunoprecipitated IP₃R immobilized on protein A-Sepharose beads. An antibody to erythrocyte ankyrin-Ab1 or a human erythrocyte ankyrin antibody (ankyrin-Ab2).

**FIG. 7. Precipitation of immunoreactive ankyrin protein by IP₃R antibody.** Triton X-100-solubilized microsomal extracts (100 μg) were immunoprecipitated as described under "Experimental Procedures" with 100 μg of either IP₃R (lane I) or erythrocyte ankyrin antibody (lane A). The immunoprecipitates together with 2 μg of rat erythrocyte ghost protein (lane G) and 20 μg of microsomal extract (lane E) were electrophoresed on 5% SDS gels and transferred to nitrocellulose. The immunoblots were probed with 1:500 dilutions of the chick erythrocyte ankyrin antibody used in the immunoprecipitation (ankyrin-Ab1) or a human erythrocyte ankyrin antibody (ankyrin-Ab2).

**FIG. 8. Removal of immunoreactive ankyrin by alkali-treatment of microsomal membranes.** Microsomal membranes (2.5 mg of protein) were incubated at 1 mg/ml in 0.1 M NaOH for 30 min at 4 °C. The entire sample was centrifuged at 100,000 x g through a sucrose barrier as described by Davis and Bennett (40). The pellet was washed twice in isolation buffer and solubilized with Triton X-100 as described under "Experimental Procedures." Detergent extracts of untreated (C) and alkali-treated (A) membranes were quenched in SDS sample buffer and electrophoresed. The Western blots were visualized with enhanced chemiluminescence, and the amounts of protein loaded on the gel were 5 μg for IP₃R antibody and 15 μg for ankyrin antibody.

cerebellar homogenates with cytochalasin B using the experimental conditions of Rossier et al. (23) did not alter the distribution of the IP₃R in cerebellar membranes or influence their detergent solubility (data not shown). In permeabilized mast cells, treatment with microtubule-disrupting agents (but not cytochalasin D) has been reported to inhibit IP₃-mediated Ca²⁺ release (24).

In the present study, we have shown that an antibody against the COOH terminus of the Type I IP₃R can immunoprecipitate 90% of the [³H]IP₃-binding sites from a detergent extract of cerebellum membranes (Fig. 5A). The cerebellum is believed to contain mRNA encoding two and possibly three distinct IP₃ receptors (8, 10), none of which share the same COOH terminus as the Type I IP₃R. Assuming the antibody used in this study is specific to the Type I IP₃R, the immunoprecipitation data would suggest that other types of IP₃ receptors contribute <10% of the ligand binding and/or are associated with Type I receptors to form heterotetramers. We also show in the present study that it is possible to measure ligand binding to immunoprecipitated IP₃R immobilized on protein A-Sepharose beads. An antibody to erythrocyte ankyrin-Ab1 or a human erythrocyte ankyrin antibody (ankyrin-Ab2).

**FIG. 9. Removal of immunoreactive ankyrin from IP₃R antibody immunoprecipitates by boiling and treatment with SDS.** Microsomal extracts (100 μg) were immunoprecipitated with IP₃R antibody. The immunoprecipitates were either washed and quenched in SDS sample buffer (1) or resuspended and boiled for 5 min in 100 μl of a buffer containing 1% SDS (w/v), 50 mM Tris-HCl, pH 8.3, 5 mM EDTA. After removal of the protein A-Sepharose beads, the supernatants were diluted to 1 ml with solubilization buffer (minus dithiothreitol) and reimmunoprecipitated with IP₃R antibody (2). The immunoprecipitates were probed on Western blots with IP₃R or erythrocyte ankyrin antibody. The amount of protein loaded in each lane was equivalent to that immunoprecipitated from 20 μg of extract for IP₃R antibody and 80 μg of extract for ankyrin antibody.

**FIG. 10. The effect of the peptide corresponding to the COOH terminus of the IP₃R on the recognition properties of IP₃R and ankyrin antibodies.** A, detergent extracts were immunoprecipitated with IP₃R antibody, and the immunoprecipitates were probed with IP₃R or ankyrin antibody after transfer to nitrocellulose. Where present, the indicated concentration of peptide corresponding to the COOH terminus of the IP₃R was preincubated with the antibodies for 1 h at 4 °C before use. The amount of protein loaded in each lane was equivalent to that immunoprecipitated from 20 μg of extract for IP₃R antibody and 80 μg of extract for ankyrin antibody. B, [³H]IP₃ binding to IP₃R or ankyrin antibody immunoprecipitates were measured as described in Table 1 in the presence or absence of 100 μg/ml of the COOH terminus IP₃R peptide. The data shown are the mean ± S.E. of three experiments.
erythrocyte ankyrin was also able to immunoprecipitate a fraction of IP$_3$ receptors from detergent extracts of cerebellar membranes as measured by ligand binding assay or Western blotting with IP$_3$R antibody. The following pieces of evidence suggest that this may be due to an association of ankyrin with the IP$_3$R rather than cross-reactivity of the ankyrin antibody with the IP$_3$R protein: 1) the reactivity of ankyrin antibody was removed by preadsorption to erythrocyte ghost membranes; 2) only a limited portion of the IP$_3$R (approximately 18%) was immunoprecipitated by maximal amounts of ankyrin antibody; 3) ankyrin immunoreactive polypeptides present in IP$_3$R antibody immunoprecipitates were selectively removed after boiling in SDS-containing buffers; 4) alkaline treatment of cerebellar membranes removed immunoreactive ankyrin without affecting the IP$_3$R. In a recent communication, Davis and Bennett (47) have reported that a 270-kDa polypeptide cross-reactive with IP$_3$R antibody is one of several proteins in brain membrane extracts adhering to an ankyrin affinity column. These data strongly support the view that the IP$_3$R can interact with ankyrin.

Low speed membranes have higher amounts of Triton X-100-insoluble binding sites and therefore it may be anticipated that there would be differences in the amount of IP$_3$R-binding sites immunoprecipitated by ankyrin antibody from detergent extracts of low speed membranes and cerebellum microsomes. This was not the case (data not shown). Experimental circumstances may dictate that a relatively constant proportion of IP$_3$R-ankyrin complexes are solubilized by detergents. However, it is also possible that binding of the IP$_3$R to ankyrin and the presence of the IP$_3$R in the Triton X-100-insoluble membrane fraction are two unrelated phenomena.

Ankyrin has a well established role as an adaptor protein connecting several integral plasma membrane proteins with the spectrin-based cytoskeleton (48, 49). Ion-translocating proteins known to associate with ankyrin include the anion-exchanger (50), voltage-dependent Na$^+$ channel (51), amiloride-sensitive Na$^+$ channel (52), and the Na$^+$/K$^+$ ATPase (53). It is also of interest that the CGMP-gated cation channel of photoreceptors is associated with a high molecular weight protein that is related to spectrin (54). Association of ankyrin with intracellular membrane proteins has not been widely documented although erythrocyte ankyrin antibodies have been reported to label internal structures (possibly longitudinal sarcoplasmic reticulum) in skeletal muscle (55). Molecular cloning studies have revealed the presence of multiple ankyrin isoforms derived from different genes and from alternative splicing of mRNA (48, 49). Both brain (39) and erythrocyte (55, 56) ankyrins possess 22 repeats of a 33-amino-acid sequence motif that has been found in several proteins, including certain transcription factors (48, 57). This sequence motif is believed to be responsible for mediating protein-protein interactions. Of the two erythrocyte ankyrin antibodies used in this study, only one was capable of immunoprecipitating IP$_3$R and recognizing specific polypeptides in brain including a band of approximately 230 kDa. Since both antibodies recognized the erythrocyte homolog of ankyrin (~210 kDa) in cerebellum extracts, it follows that this species of ankyrin is unlikely to be associated with the IP$_3$R. A conclusive identification of the ankyrin species involved is presently not possible because of the lack of availability of antibodies recognizing brain isoforms of this protein.

Attachment to ankyrin potentially allows the IP$_3$R to interact with many components of the neuronal cytoskeleton. These interactions may serve a structural role in anchoring Ca$^{2+}$ stores to the plasma membrane via the subplasmalemmal cytoskeleton (47) or may serve to restrict a pool of IP$_3$R to some other specific region of the neuron. Modulation of these interactions may also play a functional role in communicating the filling state of the intracellular store to Ca$^{2+}$ transport systems on the plasma membrane. Such mechanisms are required to explain the apparent dependence of plasma membrane Ca$^{2+}$ entry on intracellular Ca$^{2+}$ mobilization seen in many experimental Ca$^{2+}$-mobilizing stimuli (58). Disruption of cells and solubilization of membranes may perturb associations of the IP$_3$R with many regulatory or structural proteins that are potentially physiologically relevant. High affinity associations with ankyrin survive detergent solubilization, as is the case in complexes of ankyrin with several other membrane proteins (31). Further work will be required to clarify the functional significance of the attachment of the IP$_3$R to the cytoskeleton.

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**REFERENCES**

IP₃R and the Cytoskeleton