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Solubilization and Partial Characterization of Inositol 1,4,5-Trisphosphate Receptor of Bovine Adrenal Cortex Reveal Similarities with the Receptor of Rat Cerebellum

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SUMMARY

Inositol 1,4,5-trisphosphate ($InsP_3$) is an intracellular messenger generated upon stimulation of a wide variety of cells by Ca^{2+}-mobilizing stimuli. Specific binding sites for $InsP_3$ have been identified in the adrenal cortex and many other tissues. The purpose of the present study was to solubilize and further characterize $InsP_3$ receptors of bovine adrenal cortex. When adrenal cortex microsomes were incubated with Triton X-100 (1%) for 45 min and centrifuged at 100,000 × *g* for 1 hr, substantial $InsP_3$-binding activity was recovered in the pellet fraction (82 ± 46 fmol/mg of protein; K_d of 2.7 ± 1.2 nM), suggesting a possible association with the cell skeleton. Similar results were also obtained with a microsomal preparation of rat cerebellum. On the other hand, the supernatant fraction also displayed important $InsP_3$-binding activity (188 ± 67 fmol/mg of protein; K_d of 10.4 ± 2.2 nM). $InsP_3$ binding in both fractions was inhibited by heparin and was increased upon pH elevations from 6 to 9. These are two characteristic properties of $InsP_3$ receptors. Solubilized $InsP_3$ receptors displayed a molecular size around 1,000,000, as estimated by gel filtration through Sepharose-4B column. Radiation inactivation analyses of the receptors of bovine adrenal cortex and rat cerebellum revealed unusual inactivation curves, indicating binding domains of M_r 65,000, much smaller than the smallest covalent structure (subunit) of M_r 260,000 estimated by gel electrophoresis. These results suggest that the binding domain of the receptor behaves independently from the rest of the molecule and that a direct hit on the domain is needed for inactivation. Our data show that the binding sites for $InsP_3$ in the adrenal cortex have properties similar to those of sites recently purified from rat cerebellum, and they suggest that $InsP_3$ receptors from both sources might be the same molecular entity.

$InsP_3$ is an intracellular messenger generated from the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C in the response of a wide variety of cells to Ca^{2+}-mobilizing stimuli (for review, see Refs. 1–3). $InsP_3$ releases Ca^{2+} from a nonmitochondrial store that is part of the endoplasmic reticulum (4). Structure-activity studies on the ability of inositol phosphates to release Ca^{2+} suggested that $InsP_3$ was acting on a receptor (4). This idea was supported by the demonstration of binding sites for $InsP_3$ in the adrenal cortex (5, 6) and many other tissues (7–10). An $InsP_3$-binding protein was purified from rat cerebellum membranes (11) and was recently reconstituted into lipid vesicles (12). This protein was shown to mediate $InsP_3$-induced Ca^{2+} fluxes, indicating that it contained both an $InsP_3$ recognition site and a cation channel. Two other recent studies, using molecular biology approaches, permitted the identification of the sequence of the $InsP_3$ receptor protein of mouse cerebellum (13, 14). These studies revealed that the $InsP_3$ receptor had some similarities with the ryanodine receptor of skeletal muscle sarcoplasmic reticulum. In peripheral tissues (adrenal, liver, pituitary), where intracellular Ca^{2+} regulation has been more extensively studied, the $InsP_3$ receptor has a higher affinity (K_d of 1–5 nM) and a lower density (B_{max} of 10–100 fmol/mg of protein) (6, 7, 9, 15) than in cerebellum (K_d of approximately 50 nM and B_{max} of approximately 20 pmol/mg of protein) (8, 10). The inhibitory effect of low Ca^{2+} concentrations on $InsP_3$ binding to cerebellum microsomes (8, 16) has never been observed in peripheral tissues. These different properties suggested that the $InsP_3$ receptor of peripheral tissues might be different from the one of cerebellum. On the other hand, $InsP_3$ receptors of cerebellum and peripheral tissues have several similarities, including the same order of affinity for inositol phosphates and $InsP_3$ analogs, the same sensitivity to pH variation, the same affinity for heparin, and the same susceptibility to heat, trypsin, and alkylating reagents (5–10, 16–18). In the present study, we have used gel filtration and radiation inactivation analyses to further compare the

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ABBREVIATIONS: $InsP_3$, inositol 1,4,5-trisphosphate; EGTA, [ethylene bis(oxyethylenenitrilo)] tetraacetic acid.

properties of $InsP_3$ receptors of rat cerebellum and bovine adrenal cortex. Our results show that the receptors of the two tissues cannot be distinguished on the basis of their molecular size.

Experimental Procedures

Materials. [^{3}H]$InsP_3$ (20–40 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). $InsP_3$ was from Calbiochem (La Jolla, CA). ^{125}I-Angiotensin II (400 Ci/mmol) was prepared with Iodogen and ^{125}INa, as previously described (19). Triton X-100 was from Schwarz/Mann Biotech (Cleveland, OH). Heparin (ammonium salt; no. H-0880) and bovine γ-globulins (no. G-5009) were from Sigma (St. Louis, MO). Sepharose CL-4B and the gel filtration calibration kit for molecular weight determination of high molecular weight proteins were from Pharmacia LKB (Uppsala, Sweden). Anion exchange AG 1-X8 resin (formate form) was from Bio-Rad Laboratories (Richmond, CA).

Preparation of microsomes. Bovine adrenal cortices (dissected free of medullary tissue) were homogenized with eight strokes of a Dounce homogenizer (loose pestle) in a medium containing 110 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 25 mM Tris·HCl, pH 7.2, 5 mM KH_2PO_4, 1 mM dithiothreitol, and 2 mM EGTA. After stirring for 5 min and centrifugation at 500 × *g* for 15 min, the supernatant was centrifuged at 30,000 × *g* for 20 min. The pellet was resuspended in the same medium without EGTA, at a concentration of 20–30 mg of protein/ml. The proteins were measured by the method of Lowry *et al.* (20), with bovine serum albumin as the standard. The microsomes were aliquoted and stored at −70° until used.

Rat cerebella were homogenized with eight strokes of a Dounce homogenizer (tight pestle), in the same medium used for adrenal microsome preparation. After filtration through four layers of cheesecloth and centrifugation at 35,000 × *g* for 20 min, the pellet was resuspended in the same medium without EGTA, at a concentration of 10 mg of protein/ml. The microsomes were stored at −70° until used.

Solubilization of microsomes. Bovine adrenal cortex microsomes or rat cerebellum microsomes were resuspended at a concentration of 10 mg of protein/ml, in a medium containing 25 mM Tris·HCl, pH 8.5, 100 mM KCl, 20 mM NaCl, 5 mM KH_2PO_4, 0.1% bovine serum albumin, 1 mM EDTA, and 0–1.5% (v/v) Triton X-100. After 45 min at 0°, insoluble material was precipitated by centrifugation at 100,000 × *g* for 1 hr. The pellets and the supernatants were immediately tested for their $InsP_3$-binding activity.

$InsP_3$ binding assay. Intact microsomes or insoluble pellets were incubated in a medium containing 25 mM Tris·HCl, pH 8.5, 100 mM KCl, 20 mM NaCl, 5 mM KH_2PO_4, 1 mM EDTA, and 0.1% bovine serum albumin. Incubations with [^{3}H]$InsP_3$ (~1 nM) and varying concentrations of nonradioactive ligand(s) were for 30 min at 0°. Nonspecific binding was determined in the presence of 1 μM $InsP_3$. Incubations were terminated by vacuum filtration through presoaked glass fiber filters (Whatman 934-AH) and rapid washing with 2.5 ml of cold medium. The receptor-bound radioactivity was analyzed by liquid scintillation counting.

$InsP_3$ binding to solubilized proteins was measured by incubation of aliquots of the supernatants (diluted to 0.1%, v/v, Triton X-100) with [^{3}H]$InsP_3$, in a final volume of 400 μl, under the same conditions described above. After 15 min at 0°, 200 μl of 0.5% γ-globulin were added and the proteins were precipitated by addition of 600 μl of 30% polyethylene glycol (M_r 6000) and centrifugation at 5000 × *g* for 15 min. The supernatant was aspirated and the pellet-bound radioactivity was analyzed by liquid scintillation counting.

Radiation inactivation analysis. Frozen microsomes (333 μg of cerebellar protein or 5 mg of adrenocortical protein) in 1 ml of assay buffer were irradiated at −78.5°, in crushed dry ice, in a ^{60}Co irradiator at a dose rate of 1.3 Mrad/hr (Gammacell model 220; Atomic Energy of Canada, Ottawa, Ontario, Canada). After exposure to radiation for increasing periods of time, samples were stored on solid CO_2 for shipping. They were then thawed for the binding assay and the logarithm of residual binding activity was plotted as a function of dose. The radiation inactivation size of the functional receptor was calculated according to the empirical relationship (21) of $\log_{10}$ radiation inactivation size = $5.89 - \log_{10} D_{37,t} - 0.0028t$, where $D_{37,t}$ is the radiation dose in Mrad necessary to reduce the activity to 37% of the unirradiated level and t is the temperature in degrees Celsius.

Other methods. ^{125}I-Angiotensin II binding was performed for 45 min at room temperature in a buffer containing 20 mM Tris·HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% bovine serum albumin, and 50,000 cpm of ^{125}I-angiotensin II. The bound and free ligand was separated by vacuum filtration through glass fiber (934-AH) filters. Alkaline phosphatase activity was measured according to the method of Colbeau and Maroux (22), with *p*-nitrophenylphosphate as substrate. $InsP_3$5-phosphatase activity was measured as previously described (6, 18).

Results

Extraction of membrane proteins with Triton X-100. Bovine adrenal cortex microsomes were treated with detergent solutions for 45 min at 0° and centrifuged at 100,000 × *g* for 1 hr. The particulate fractions were examined for $InsP_3$ binding, angiotensin II binding, and protein content. As shown in Table 1, although $InsP_3$ binding decreased substantially in the particulate fractions after treatment with detergent, it was not completely abolished even after extraction with concentrations of Triton X-100 as high as 1.5%. About 45% of the membrane proteins were extracted with 0.3% Triton X-100 and 72% with 1.5% Triton X-100. The efficacy of the detergent was also confirmed by its ability to solubilize the angiotensin II receptor (a known integral membrane protein of bovine adrenal cortex). Our microsomal preparation contained high angiotensin II-binding activity [K_d of 2 nM and B_{max} of 1.5 pmol/mg of protein, consistent with a previous report, (23)]. The receptors were apparently completely extracted with 0.3% Triton X-100 [also consistent with a previous report (19)].

Because substantial $InsP_3$-binding activity was still present in Triton X-100-extracted microsomes, we decided to further characterize this residual activity. Dose-displacement curves shown in Fig. 1 indicate that [^{3}H]$InsP_3$ binding to intact microsomes or 1% Triton X-100-extracted microsomes was competitively inhibited by increasing concentrations of unlabeled $InsP_3$. Scatchard plots derived from these data indicate a decrease of affinity (K_d = 0.9 nM) and of maximal binding

TABLE 1

$InsP_3$ and angiotensin II binding and protein content of Triton X-100-extracted microsomes

Adrenal cortex microsomes (10 mg of protein/ml) were incubated for 45 min at 0° with different concentrations of Triton X-100. After centrifugation, the pellets were resuspended in water and aliquots were analyzed for their $InsP_3$ and angiotensin II binding activities and their protein content. $InsP_3$ binding at 100% represents specific binding (~1,500 cpm) to intact microsomes (1 mg of protein) incubated with [^{3}H]$InsP_3$ (15,000 cpm; 1 nM). Angiotensin II binding at 100% represents specific binding (~2200 cpm) to intact microsomes (25 μg of protein) incubated with ^{125}I-angiotensin II (40,000 cpm; 0.5 nM). Each value is the mean ± standard deviation of data from at least three different experiments.

Triton X-100 (v/v)	$InsP_3$ binding	Angiotensin II binding	Protein content
%	%	%	*mg/ml*
0	100	100	10
0.3	63 ± 12	ND[a]	5.6 ± 0.9
1.0	46 ± 18	ND	3.5 ± 0.4
1.5	34 ± 13	ND	2.8 ± 0.4

[a] ND, not detectable.

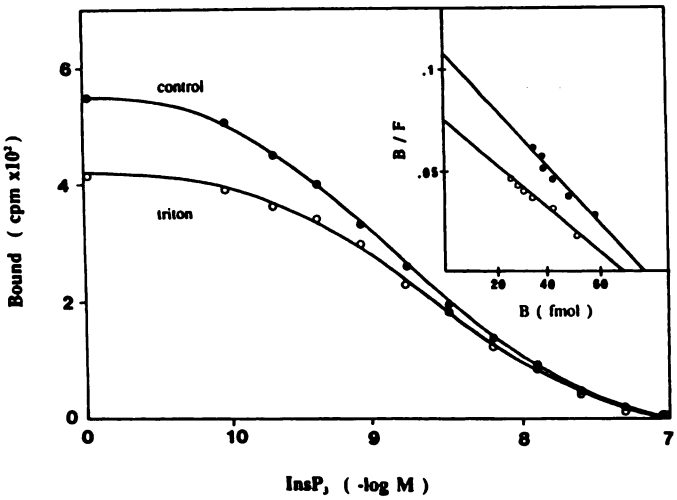

Fig. 1. $InsP_3$ binding to Triton X-100-extracted microsomes. Intact microsomes (1 mg of protein) or 1% Triton X-100-extracted microsomes (equivalent to 1 mg of protein of intact microsomes) were incubated at 0°, for 30 min, in the presence of [^{3}H]$InsP_3$ (18,000 cpm, ~1.5 nM) and increasing concentrations of unlabeled $InsP_3$. Specific binding to intact (●) and detergent-extracted (○) microsomes was measured as indicated in Experimental Procedures. *Inset*, Scatchard representation of the same experimental data. Each *point* is the mean of duplicate determinations. This typical experiment is representative of 11 experiments that gave similar results. *B*, bound; *B/F*, bound/free.

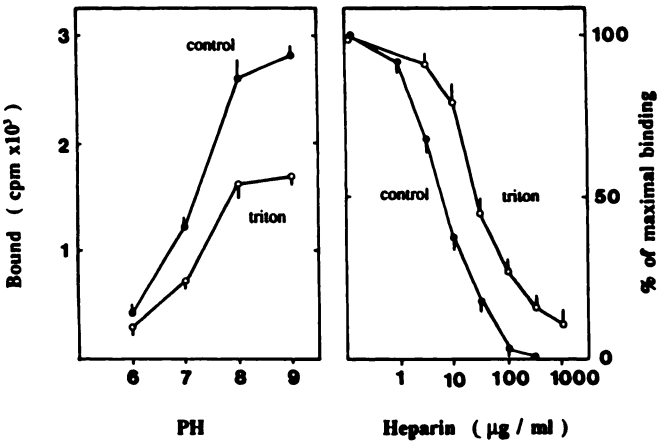

Fig. 2. Effects of pH and heparin on $InsP_3$ binding to Triton X-100-extracted microsomes. *Left*, intact microsomes (1 mg of protein) or 1% Triton X-100-extracted microsomes (equivalent to 1 mg of protein of intact microsomes) were incubated at 0°, for 30 min, in the presence of [^{3}H]$InsP_3$ under different pH conditions. Specific binding to intact (●) and detergent-extracted (○) microsomes was measured as indicated in Experimental Procedures. *Right*, intact microsomes (●) or 1% Triton X-100-extracted microsomes (○) were incubated in the presence of [^{3}H]$InsP_3$ and increasing concentrations of heparin. These experiments, performed in triplicate (mean ± standard deviation), are representative of two similar experiments.

TABLE 2

Binding parameters of membrane fractions

Adrenal cortex microsomes (10 mg of protein/ml) were incubated for 45 min at 0° with different concentrations of Triton X-100. After centrifugation at 100,000 × *g* for 1 hr, $InsP_3$-binding activity in the residual pellets and the supernatants was measured by Scatchard analyses. Each value is the mean ± standard deviation of data from the indicated number of experiments.

	K_d	B_{max}	Number of experiments
	nM	*fmol/mg*	
Intact membranes	1.9 ± 0.9	135 ± 54	12
0.3% Triton X-100 pellet	2.1 ± 0.6	108 ± 49	5
1% Triton X-100 pellet	2.7 ± 1.2	82 ± 46	11
1.5% Triton X-100 pellet	2.7 ± 1.3	63 ± 41	6
1% Triton X-100 supernatant	10.4 ± 2.2	188 ± 67	3

capacity (68 fmol/mg of protein) in 1% Triton X-100-extracted microsomes, as compared with intact microsomes (K_d of 0.7 nM and B_{max} of 79 fmol/mg of protein). Similar results indicating that Triton X-100-extracted microsomes still conserve substantial $InsP_3$-binding activity were obtained with many different microsomal preparations (Table 2). Increasing concentrations of Triton X-100 decreased both the affinity and the number of binding sites for $InsP_3$ in the residual pellets. It was important to verify that the binding sites for $InsP_3$ in Triton X-100-extracted microsomes were the same entities as those characterized in intact microsomes and not unrelated binding sites unmasked upon removal of membrane lipids. It has previously been shown that heparin potently inhibits $InsP_3$ binding to membrane fractions of brain (8) and adrenal cortex (18). Fig. 2 (*right*) shows that heparin competitively inhibited the binding of $InsP_3$ to intact (IC_{50} ~ 8 µg/ml) and detergent-extracted (IC_{50} ~ 20 µg/ml) microsomes. Another peculiar property of $InsP_3$ receptors is their sensitivity to pH variations. $InsP_3$ binding increases gradually as the pH is raised from 6 to 9 (8, 17). This property was also observed in 1% Triton X-100 extracted microsomes, as shown in Fig. 2 (*left*). These results suggest that the residual binding sites for $InsP_3$ in Triton X-100-extracted microsomes of bovine adrenal cortex are the same entities as those detected in intact microsomes.

Soluble $InsP_3$-binding activity. Our results indicated that some $InsP_3$ binding activity was extracted with high concentrations of Triton X-100. Substantial binding activity was recovered in the supernatant of 1% Triton X-100-extracted microsomes. Fig. 3, (*upper*) shows that [^{3}H]$InsP_3$ binding was competitively inhibited by increasing concentrations of unlabeled $InsP_3$. Tracer binding was reduced by 10% in the presence of 2 nM unlabeled $InsP_3$ and was completely inhibited by 1 µM $InsP_3$. Scatchard analysis of the binding data was consistent with a single set of sites, with a K_d of 10.3 nM and maximal binding capacity of 239 fmol/mg of protein (Fig. 3, *upper*, *inset*). These experiments showed unexpectedly large amounts of $InsP_3$ binding sites in the supernatant fraction. The total amount of binding sites in the supernatant and the pellet of 1% Triton X-100-extracted microsomes corresponds to about twice the number of binding sites detected in intact microsomes (Table 2). These results suggest that intact microsomes contain a number of undetectable receptors that are solubilized or unmasked after treatment with Triton X-100. We verified that $InsP_3$ binding sites detected in the supernatant fraction had the same properties as those present in intact microsomes. Heparin was able to inhibit the binding of $InsP_3$ to solubilized receptors (Fig. 3, *lower left*) and the binding activity at pH 8.5 was greater than at pH 6.5 (Fig. 3, *lower right*).

$InsP_3$ receptor of rat cerebellum. Dose displacement of [^{3}H]$InsP_3$ binding to a microsomal preparation of rat cerebellum (Fig. 4, *upper*) revealed high affinity binding sites with a K_d of 10.2 ± 6.1 nM and maximal binding capacity of 5.1 ± 2.2 pmol/mg of protein (mean ± SD of eight experiments). After extraction with 1% Triton X-100, cerebellum microsomes still contained a substantial amount (0.9 ± 0.6 pmol/mg of protein) of high affinity binding sites (4.6 ± 3.7 nM, mean ± SD of four

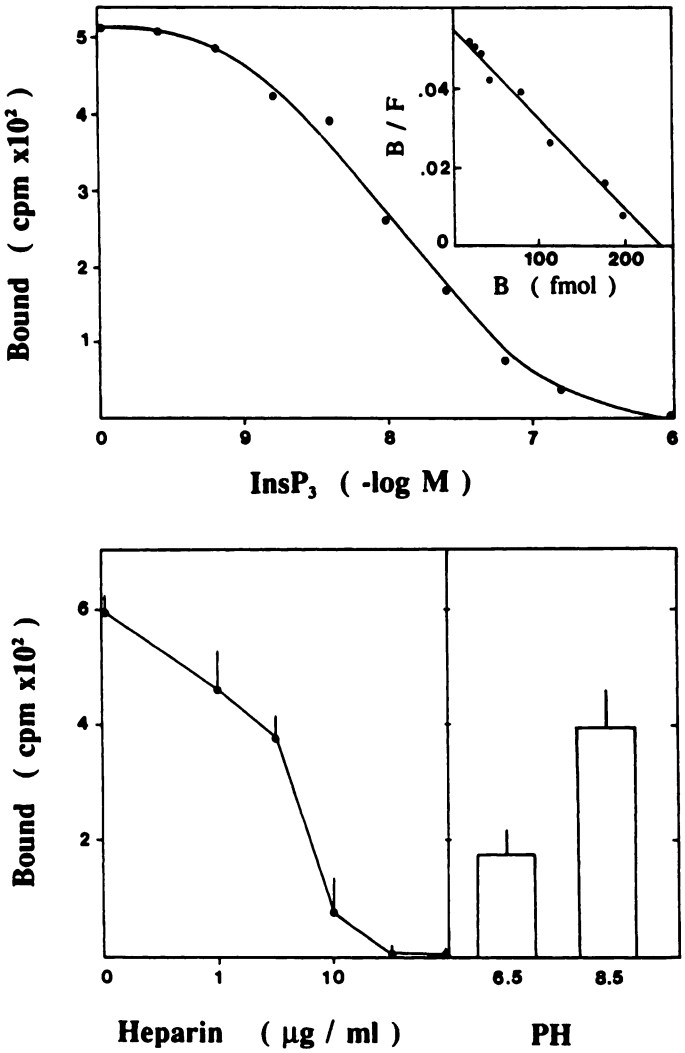

Fig. 3. $InsP_3$ binding to solubilized receptors. *Upper*, supernatant fractions (equivalent to 1 mg of protein of intact microsomes) of 1% Triton X-100-treated microsomes were incubated at 0°, for 15 min, in the presence of [^{3}H]$InsP_3$ (10,500 cpm) and increasing concentrations of unlabeled $InsP_3$. Bound and free ligand was separated by polyethylene glycol precipitation, as described in Experimental Procedures. Total binding to the protein pellet was 1014 cpm and nonspecific binding, in the presence of 1 µM $InsP_3$ was 506 cpm. *Inset*, Scatchard analysis of the same binding data. *B*, bound; *B/F*, bound/free. These results, performed in duplicate, are representative of three similar experiments. *Lower left*, supernatant fractions were incubated in the presence of increasing concentrations of heparin, and [^{3}H]$InsP_3$ binding was measured as described above. These results, performed in triplicate (mean ± standard deviation), are representative of two similar experiments. *Lower right*, $InsP_3$ binding to supernatant fractions was measured under two different pH conditions. The results, performed in triplicate (mean ± standard deviation), are representative of three similar experiments.

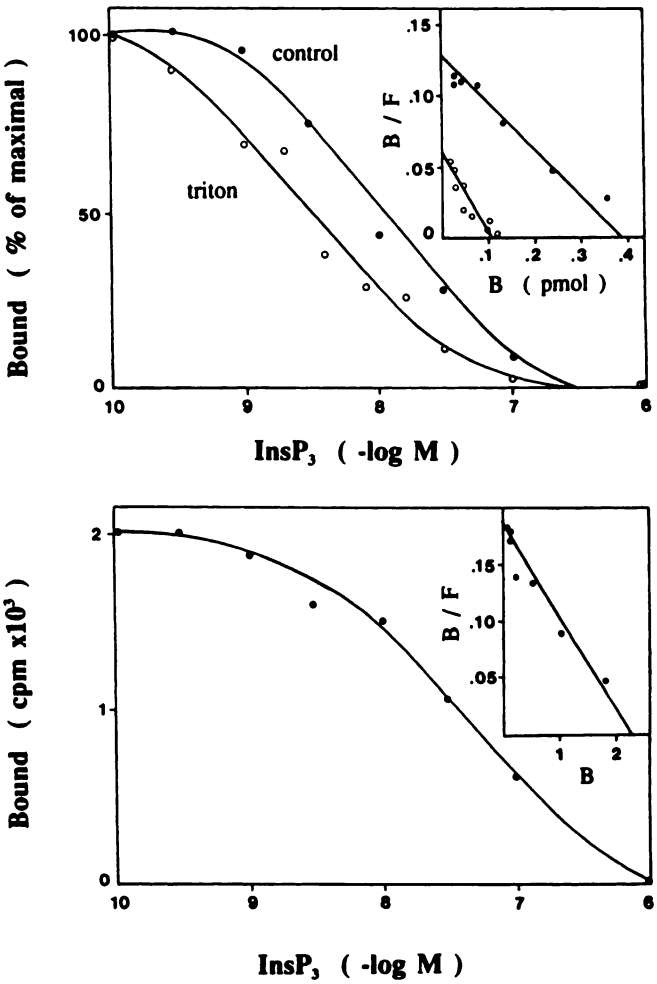

Fig. 4. $InsP_3$ receptor of rat cerebellum. *Upper*, rat cerebellum microsomes (50 µg of protein) or 1%-Triton X-100-extracted microsomes (equivalent to 200 µg of protein of intact microsomes) were incubated at 0°, for 30 min, in the presence of [^{3}H]$InsP_3$ (12,000 cpm) and increasing concentrations of unlabeled $InsP_3$. Specific binding to intact microsomes (●) was 1016 cpm (100%) and to detergent-extracted microsomes (○) was 594 cpm (100%). *Inset*, Scatchard analyses of the same binding data. *B*, bound; *B/F*, bound/free. These results, performed in duplicate, are representative of eight similar experiments. *Lower*, supernatant fractions (equivalent to 200 µg of protein of intact microsomes) of 1% Triton X-100-extracted microsomes were incubated with [^{3}H]$InsP_3$ (13,000 cpm) and increasing concentrations of unlabeled $InsP_3$. Specific binding was measured as indicated in Experimental Procedures. *Inset*, Scatchard analysis of the same data [bound/free versus bound (pmol)]. This experiment, performed in duplicate, is representative of three similar experiments.

experiments). The supernatant fraction of 1% Triton X-100-extracted cerebellum microsomes contained high $InsP_3$-binding activity (Fig. 4, *lower*). The maximal binding capacity was 12.5 ± 5.3 pmol/mg of protein and the affinity was 41 ± 15 nM (mean ± SD of three experiments). Although Triton X-100 showed a higher solubilizing efficacy in this preparation, the results obtained with rat cerebellum are consistent with those obtained with bovine adrenal cortex. It is interesting to note that, with both preparations, the number of binding sites found in the supernatant fraction was higher than the number found in intact microsomes.

Gel filtration of solubilized $InsP_3$ receptor. The supernatant fraction of 1% Triton X-100-treated adrenal cortex microsomes was applied to the top of a Sepharose column. $InsP_3$-binding activity eluted as a broad peak with an elution volume of 44 ml (Fig. 5). As observed for the $InsP_3$ receptor of rat cerebellum (11), the peak eluted somewhat earlier than thyroglobulin (elution volume = 49 ml), indicating a Stokes radius of approximately 10 nm. For a globular protein, this would correspond to a molecular weight of 853,000 ± 81,000 (mean ± SD of three experiments). This value does not take into account any size increment due to Triton X-100 bound to the protein. A minor peak of $InsP_3$-binding activity eluted with blue dextran. This peak may represent aggregated receptors, because it corresponds to about twice the size of a single

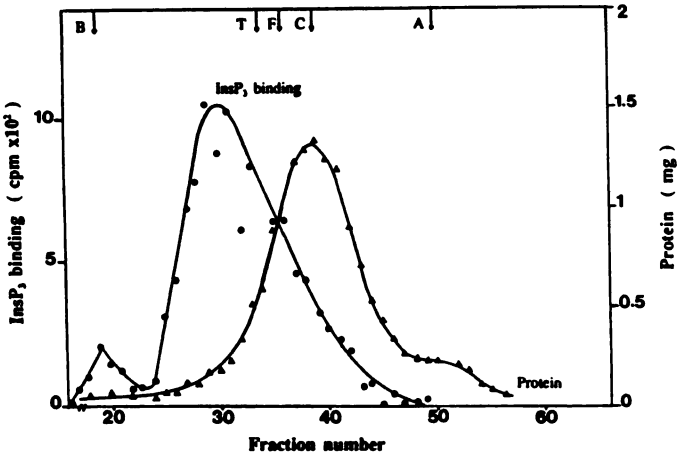

Fig. 5. Gel filtration of solubilized $InsP_3$ receptor. The supernatant fraction (1.5 ml) of 1% Triton X-100-treated adrenal cortex microsomes was applied to the top of a Sepharose CL-4B column (15 mm × 350 mm) and eluted with a buffer containing 0.1% Triton X-100. Each fraction (1.5 ml) was evaluated for its $InsP_3$-binding capacity (as described in Experimental Procedures) and protein content [by the method of Lowry *et al.* (20)]. *Arrows*, the elution volume of molecular weight markers: *B*, blue dextran (M_r 2,000,000); *T*, thyroglobulin (M_r 669,000); *F*, ferritin (M_r 440,000); *C*, catalase (M_r 232,000); *A*, ^{125}I-angiotensin II (M_r 1,000). This typical elution profile is representative of three experiments, which gave similar results.

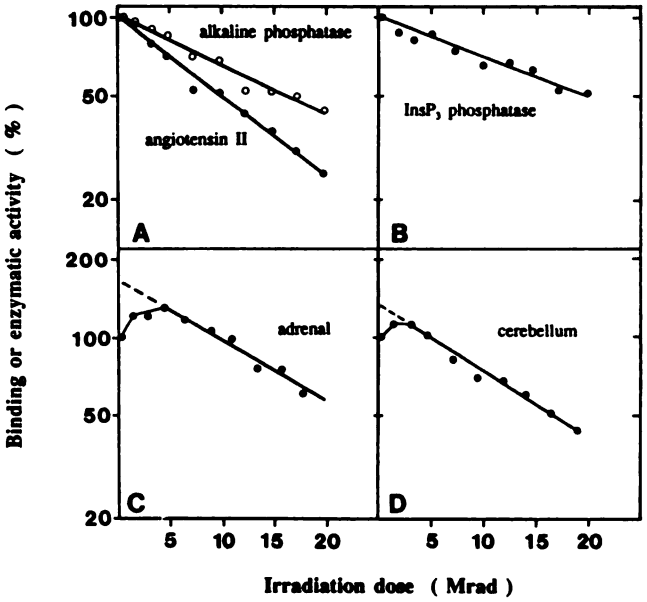

Fig. 6. Target size analysis. Effect of irradiation on different activities found in microsomal preparation of bovine adrenal cortex or rat cerebellum. The results of typical experiments are expressed as mean of triplicate determinations. *100%*, specific binding or enzymatic activity measured in unirradiated microsomes. For angiotensin II binding (A), 100% = 6,480 cpm bound/50 μg of protein. For alkaline phosphatase activity (A), 100% = 1.05 A_{420} units/12 min/100 μg of protein. For $InsP_3$ phosphatase activity (B), 100% = 0.65 nmol of $InsP_3$ hydrolyzed/20 min/20 μg of protein. For $InsP_3$ binding to adrenal microsomes (C), 100% = 1240 cpm bound/mg of protein. For $InsP_3$ binding to cerebellum microsomes (D), 100% = 789 cpm bound/100 μg of protein. Each *curve* is representative of at least three similar experiments with different microsomal preparations. In the cases where the lines did not intercept at 100% (C and D) D_{37} values were calculated from the extrapolated intercept.

receptor. The great bulk of proteins (Fig. 5) eluted in fractions 32–45, corresponding to molecular size in the range of 600,000 to 40,000. If this filtration technique was used as the first step for purification of the $InsP_3$ receptor, collection of fractions 24 to 35, which contain 77% of $InsP_3$-binding activity and 24% of total proteins, would represent a roughly 3-fold purification step. The recovery of total proteins [estimated by the method of Lowry *et al.* (20)] varied between 80 and 90% among the experiments.

Radiation inactivation studies. To determine the functional size of the $InsP_3$ receptor of bovine adrenal cortex and rat cerebellum, we employed the radiation inactivation technique. This technique allows estimation of molecular masses of membrane proteins *in situ*, without need for detergent solubilization. The sizes of the angiotensin II receptor, alkaline phosphatase, and $InsP_3$ 5-phosphatase of bovine adrenal cortex were also estimated to establish the validity of the method. For the angiotensin II receptor, the inactivation curve (Fig. 6A) gave a molecular size of 90,100 ± 10,800 (mean ± SD of four experiments). This value is similar to that reported for the rat liver angiotensin II receptor (M_r 93,000), estimated by gel filtration (19), and for the rat adrenal angiotensin II receptor (M_r116,000), estimated by affinity cross-linking (24) and photoaffinity labeling (25). The estimated molecular size of alkaline phosphatase, used as an internal standard, was 64,100 ± 8,700 (mean ± SD of three experiments) (Fig. 6A). This is similar to the values estimated by radiation inactivation in guinea pig kidney microsomes (M_r 70,000) (26) and by gel electrophoresis in porcine intestinal mucosa (M_r 64,000) (22) and human liver (M_r 83,000) (27). The estimated molecular size of $InsP_3$ 5-phosphatase was 52,300 ± 10,200 (mean ± SD of four experiments) (Fig. 6B). This is similar to the value estimated by gel filtration in rat liver membranes (M_r 77,000) (28) and in human platelet membranes (M_r 49,000) (29). A cytosolic $InsP_3$ 5-phosphatase activity was also found in human platelets, with a molecular size of 59,000 (29) and 38,000 (30). For $InsP_3$ receptors, inactivation curves were of unusual shape (Fig. 6, C and D). $InsP_3$-binding activity was either unaffected or slightly increased by low radiation doses and was progressively diminished for doses above 5 Mrad. This phenomenon was common for preparations from cerebellum and adrenal cortex. It could be interpreted as inactivation of a large inhibitory component or as a cooperative effect between different components of a large multimeric structure. The estimated molecular sizes derived from the inactivation curves were 64,600 ± 10,500 and 65,700 ± 4,700 (mean ± SD of three experiments) for adrenal and cerebellum receptors, respectively. These values are different from that reported for the rat cerebellum $InsP_3$ receptor (M_r 260,000), estimated by gel electrophoresis (11), or from those obtained by gel filtration [M_r 1,000,000 (11) or M_r 850,000 (the present study)]. Our results suggest that the binding site is a small independent domain of the $InsP_3$ receptor protein. Most importantly, they show that $InsP_3$ receptors of bovine adrenal cortex and rat cerebellum have the same molecular target size.

Discussion

The properties of $InsP_3$ binding sites in many peripheral tissues (5–9, 17) are consistent with a role of physiological receptors in mediating the effect of $InsP_3$ on Ca^{2+} movements during cell activation. Reconstitution of purified rat cerebellum $InsP_3$ receptors into phospholipid vesicles directly demonstrated that the protein is a calcium channel (12). In the present

study, we used the same conditions as did Supattapone *et al.* (11) to solubilize $InsP_3$ receptors of bovine adrenal cortex. We showed that a good proportion of binding sites are resistant to solubilization with Triton X-100. From the same microsomal preparation, all the receptors for angiotensin II (integral membrane proteins) and 72% of total membrane proteins were extracted with Triton X-100. $InsP_3$-binding activity in detergent-extracted pellet was sensitive to pH variation from 6 to 9 and was potently inhibited by heparin. These two distinctive properties previously observed in intact microsomes (17, 18), indicate that the detergent-insoluble binding sites for $InsP_3$ are the same molecular entities identified as $InsP_3$ receptors. Resistance of $InsP_3$ receptors to solubilization with Triton X-100 is not a phenomenon restricted to bovine adrenal cortex. Our study showed that some $InsP_3$-binding activity of rat cerebellum was also resistant to solubilization with Triton X-100. The same phenomenon was also observed with rat liver membranes.[1] The effect of Triton X-100 on the dispersion of membrane lipids and on the solubilization of membrane proteins has been the object of several studies (see, for example, Refs. 31–33). These studies have shown that the Triton X-100-insoluble pellet was mainly composed of plasma membrane skeleton and possibly rough endoplasmic reticulum skeleton (31). Our results suggest that $InsP_3$ receptors (or some of them) are associated with the cytoskeleton. Whether this association is related to normal synthesis and intracellular traffic of proteins or whether it has some functional significance in the regulation of cell Ca^{2+} remains to be clarified.

Specific $InsP_3$ binding in supernatant fractions of Triton X-100-treated adrenal cortex microsomes was inhibited by heparin and was sensitive to pH. Although the binding sites had a lower affinity (10 nM) than those of intact microsomes (1.9 nM), we suppose that they represent the same molecular entities. A significant decrease of affinity (from 10 to 41 nM) was also observed upon solubilization of rat cerebellum receptors. This effect could be due to a conformational change induced by removal of surrounding lipids. Gel filtration of solubilized $InsP_3$ receptors of bovine adrenal cortex revealed a large molecular size, similar to that reported for the $InsP_3$ receptor of rat cerebellum. It was recently shown that cerebellum receptors were multimeric structures composed of four subunits of relative molecular weight of 260,000 (12–14, 34). In the adrenal cortex, the low concentration of $InsP_3$ receptors does not facilitate their purification and further characterization. However, with the radiation inactivation technique, we could evaluate the functional size of $InsP_3$ receptors. We found a value of M_r 65,000, which is much smaller than the subunit size estimated by gel electrophoresis. In rat cerebellum, however, we found an identical target size and, most interestingly, irradiation curves in both tissues showed a similar unusual shape. The significance of these biphasic curves is presently unknown and further experiments will be needed in order to clarify this phenomenon. These results, however, strongly suggest that $InsP_3$ receptors of bovine adrenal cortex are similar to those of rat cerebellum. A recent study reported that $InsP_3$ receptors in liver and cerebellum had similar molecular target sizes around 260,000 (35). The same authors also found a molecular target size of 191,000 for $InsP_3$ 5-phosphatase. The difference from our results cannot be attributed to different processing of their experimental data, because their $D_{37,t}$ value (5 Mrad) corresponds to a molecular weight of 256,000 (if calculated according to our empirical equation). It is well documented that experimental conditions such as source nature may profoundly affect molecular weight determinations by radiation inactivation analysis (see for example 21, 36, 37). This may particularly hold true for a multidomain protein such as a receptor. Depending on conditions that determine interactions between the domains inside the protein, some domains may behave quite independently as far as their biological function is concerned. Examples of such independent domains were found in immunoglobulin E and its receptor (38), in the (Na^+, K^+)-ATPase (39), and in the NADH:nitrate reductase (40, 41).

In conclusion, we have shown that $InsP_3$ receptors are not readily extracted with Triton X-100; this may indicate an association with the cell skeleton. Our major contribution was to show that the $InsP_3$ receptor of bovine adrenal cortex had a molecular size (estimated by gel filtration and by radiation inactivation) similar to that of the rat cerebellum receptor. These data suggest that the same receptor protein is present in both tissues.

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[1] G. Boulay and G. Guillemette, unpublished observations.

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