Solubilization and separation of inositol 1,3,4,5-tetrakisphosphate- and inositol 1,4,5-trisphosphate-binding proteins and metabolizing enzymes in rat brain

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The two inositol phosphate-binding proteins, the $\text{Ins}(1,4,5)\text{P}_3$ (InsP_3) and $\text{Ins}(1,3,4,5)\text{P}_4$ (InsP_4) receptors, and the two particulate InsP_3 -metabolizing enzymes, InsP_3 5-phosphatase and InsP_3 3-kinase, were solubilized with detergent from rat cerebellar membranes. These four activities are shown to be distinct molecular species by separation using a variety of protein chromatographic steps. The pharmacology of the partially purified InsP_4 -binding site indicates that the binding has a high affinity and selectivity for InsP_4 over InsP_3 . These results suggest the existence of a distinct specific InsP_4 -binding protein which may represent the receptor for this putative second messenger.

INTRODUCTION

Receptor-mediated activation of inositol lipid hydrolysis in the plasma membrane by phospholipase C is generally recognized as a signal for Ca^{2+} mobilization in many cells. One product of the hydrolysis, $\text{Ins}(1,4,5)\text{P}_3$ (hereafter InsP_3) is a modulator of Ca^{2+} levels within the cell (for reviews see [1,2]). InsP_3 releases intracellular Ca^{2+} after binding to specific intracellular membrane-associated receptors, which have been identified in a wide variety of tissues [3–11]. Recently this receptor was purified to homogeneity from brain tissue [12]. The purified InsP_3 -binding protein appears to differ from any known inositol phosphate-related enzyme. Its direct functional association with Ca^{2+} release has recently been shown in our laboratory by reconstitution of the protein into lipid vesicles and stimulation of Ca^{2+} flux selectively by InsP_3 [13].

Termination of the InsP_3 effect on Ca^{2+} mobilization can occur by two routes. InsP_3 5-phosphatase converts InsP_3 into $\text{Ins}(1,4)\text{P}_2$ [14–22], which does not appear to affect Ca^{2+} levels. The second route of metabolism is by an InsP_3 3-kinase, which phosphorylates $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,3,4,5)\text{P}_4$ (hereafter InsP_4). InsP_3 3-kinase has been described in several tissues [23–29], including brain, from which a soluble InsP_3 kinase has been purified [28]. The product of the 3-kinase enzyme, InsP_4 , has been implicated as a second messenger in several systems [30–34]. Proposed roles of InsP_4 include the modulation of intracellular Ca^{2+} through re-uptake, sequestration or other modes of regulation of the InsP_3 -sensitive Ca^{2+} pool. InsP_4 binding has been demonstrated in brain [35–36], HL 60 cells [37] and adrenal cortex [38]. This InsP_4 -binding site has not yet been shown to be distinct from metabolic enzymes or other inositol phosphate-binding proteins.

To clarify the role of InsP_4 as a second messenger, we have recently focused on identification of the InsP_4 -binding site. In the present study we have solubilized an InsP_4 -binding site, the InsP_3 receptor, a particulate InsP_3 3-kinase and a particulate InsP_3 5-phosphatase from rat brain membranes. These four proteins have been partially purified, and we have resolved the two inositol phosphate-binding activities and the two enzymic activities by chromatographic methods. In addition, the inositol

phosphate specificity of ligand binding to the partially purified binding sites was determined.

MATERIALS AND METHODS

Materials

$[^3\text{H}]\text{InsP}_3$ (17 Ci/mmol), $[^3\text{H}]\text{InsP}_4$ 17 Ci/mmol and Formula 963 scintillation cocktail were obtained from NEN/DuPont. InsP_3 , InsP_4 and other unlabelled inositol phosphates were obtained from Calbiochem. Dowex resin AGX8 (200–400 mesh; formate form) and Bio-Gel P10 were obtained from Bio-Rad. Calmodulin–Sephacrose, heparin–agarose and CHAPS were obtained from Sigma. Concanavalin A (Con A)–Sephacrose was purchased from Pharmacia. Other reagents were from Sigma.

Preparation of membranes, cytoplasmic fractions and membrane solubilization

The membranes and cytoplasmic fractions were prepared as described previously [12], with the following modifications. Cerebella from ten male Sprague–Dawley rats (200–300 g) were homogenized (Polytron setting 6, 10 s) at 4 °C in 100 ml of 50 mM-Tris/HCl, pH 7.7, containing 1 mM-EDTA, 1 mM- β -mercaptoethanol, 10 mg of phenylmethanesulphonyl fluoride, 75 μg of chymostatin and 80 μg of pepstatin. Homogenates were centrifuged (15 min; 45000 g) and the supernatant was kept. Pellets were resuspended in 100 ml of homogenization buffer containing 150 mM-NaCl and centrifuged (15 min; 45000 g). The membrane pellets were resuspended in 100 ml of homogenization buffer. The first and second supernatants were pooled, centrifuged (40 min; 100000 g), and supernatants were kept for binding and enzyme assays. Membrane proteins were solubilized by adding CHAPS (final concn. 1%) to resuspended membranes, stirring gently for 1 h, then centrifuging (45 min; 45000 g). All procedures were done at 4 °C, with 1 ml samples taken at each step for assay.

InsP_3 kinase and InsP_3 phosphatase

InsP_3 kinase was measured by incubating 5–20 μl of a sample with 50 mM-Tris/HCl, pH 8.0, containing 4 mM-ATP, 8 mM-

Abbreviation used: Con A, concanavalin A.

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Mg²⁺, 25–50 nM-InsP₃ and 0.03 µCi of [³H]InsP₃ in a final volume of 400 µl for 3–10 min. The reaction was stopped by adding 5 ml of 0.1 M-formic acid. The solution was passed over 0.5 g columns of Dowex AGX8 (200–400 mesh; formate form) and washed with 5 ml of 0.1 M-formic acid. Ins(1,4)P₂, InsP₃ and InsP₄ were eluted sequentially with 10 ml each of 0.1 M-formic acid, containing 0.4 M-, 0.8 M- and 1.0 M-ammonium formate respectively. To each 5 ml fraction was added 15 ml of scintillation cocktail (Formula 963) and radioactivity was measured in a Beckman scintillation counter.

InsP₃ phosphatase was measured in a similar manner by incubating 1–10 µl of sample in 400 µl of 50 mM-Hepes, pH 7, containing 4 mM-MgCl₂, 50 nM-InsP₃ and 0.03 µCi of [³H]InsP₃ for 1 min.

Binding assays for InsP₃ and InsP₄

Membrane binding of [³H]InsP₃ or [³H]InsP₄ was determined by incubating 50 µl of membranes (0.6 mg/ml) with 0.025 µCi of [³H]InsP₃ or [³H]InsP₄ in 500 µl of 50 mM-Tris/HCl, pH 8.3, and 1 mM-EDTA for 10 min at 0 °C. Bound ligand was separated from unbound by centrifugation for 10 min at 12000 g. Pellets were solubilized with 100 µl of 1% SDS and transferred to scintillation mini-vials; 5 ml of Formula 963 counting cocktail was added, and radioactivity determined. Non-specific binding was determined by including 10 µM unlabelled InsP₃ or InsP₄ in the assay.

Binding to cytoplasmic or detergent-solubilized fractions was assayed by spun-column chromatography [12]. Samples were incubated with 0.01 µCi of [³H]InsP₃ or [³H]InsP₄ in a total volume of 200 µl of 50 mM-Tris/HCl, pH 8, containing 0.2% Triton X-100 and 1 mM-EDTA for 10 min at 4 °C, loaded on a 1 ml column packed with Bio-Gel P10 resin (70 g/l), and centrifuged for 5 min at 1000 g. The void volume of the column was collected, 5 ml of Formula 963 counting cocktail added, and radioactivity measured. Non-specific binding was determined by including 10 µM unlabelled InsP₃ or InsP₄ in the assay.

Separation of enzyme activity

NaCl (final concn. 250 mM) was added to solubilized protein supernatants to which washed heparin-agarose (7.5 ml packed vol.) was added, incubated for 1 h on a rotator, poured into a column, and non-adherent proteins were collected. The column was washed with 100 ml of homogenization buffer containing 1% CHAPS and 250 mM-NaCl, then eluted with 15 ml of homogenization buffer containing 1% CHAPS and 750 mM-NaCl (elution buffer). The eluate was collected, the resin washed with an additional 8 ml of elution buffer, and the two eluates were pooled.

The 750 mM-NaCl heparin eluate was adjusted to 1 mM-MgCl₂ and 1 mM-CaCl₂, incubated with 3 ml of packed Con A-Sepharose for 1 h on a rotator, poured through a column, and non-adherent proteins were collected. The column was washed with 20 ml of homogenization buffer containing 1% CHAPS, 750 mM-NaCl, 1 mM-MgCl₂ and 1 mM-CaCl₂. The resin was eluted with 10 ml of homogenization buffer containing 1% CHAPS, 2 mM-EDTA and 1 M-α-methyl mannoside for at least 1 h on a rotator, and the eluate was collected. The Con A flow-through (23 ml) was further separated by incubation with calmodulin-agarose as described in ref. [28]. The Con A flow-through was concentrated 10-fold in a Centriprep-30 apparatus (Amicon), and the concentrate was diluted back 10-fold with 25 mM-Tris/HCl (pH 7.2)/1 mM-magnesium acetate/200 mM-KCl/0.2 mM-CaCl₂ in 0.1% Triton X-100 (load buffer). The sample was incubated with approx. 2–5 ml of packed calmodulin-agarose resin for 1.5 h on a rotator. The sample was poured into a column and the non-adherent protein collected.

Table 1. Regional distribution of particulate InsP₃ phosphatase and InsP₃ kinase activity in rat brain

Fresh rat brains were dissected and the tissue was homogenized, and washed with 150 mM-NaCl in homogenization buffer at approx. 1 mg of protein/ml. InsP₃ kinase and phosphatase assays were performed as described in the Materials and methods section. InsP₃ kinase was assayed for 3 min at 24 °C with 5 µg of tissue in a volume of 400 µl; 100% corresponds to a specific activity of approx. 50 pmol/min per mg of protein determined with 50 nM-InsP₃. InsP₃ phosphatase was assayed for 1 min at 24 °C with 5 µg of tissue in a volume of 400 µl; 100% corresponds to a specific activity determined with 50 nM-InsP₃. Assays were performed in triplicate. Data are mean values of three independent determinations which varied by less than 15%.

Brain region	Relative activity (%)	
	InsP ₃ phosphatase	InsP ₃ kinase
Cerebellum	100	84.2
Hippocampus	27.1	100
Striatum	31.0	100
Cortex	31.4	81.6
Midbrain	37.9	15.3
Brain stem	35.4	7.9

The column was washed with 10–20 ml of the load buffer and eluted for 2 h with 15 ml of 25 mM-Tris/HCl (pH 7.2)/1 mM-magnesium acetate/200 mM-KCl/1.0 mM-EGTA in 0.1% Triton X-100. All procedures were done at 4 °C, and 1 ml samples were taken at each step for assay.

RESULTS

Regional distribution of membrane-associated InsP₃ kinase and InsP₃ phosphatase

We have shown previously that InsP₃ receptor binding in rat brain is considerably enriched in the cerebellum [7,8]. In contrast, InsP₄ binding to membranes displays similar levels in the cerebellum, hippocampus and corpus striatum, somewhat lower levels in the midbrain and lowest levels in the brain stem and cerebral cortex [35]. In the present study we have determined the regional distributions of membrane-associated InsP₃ 3-kinase and InsP₃ 5-phosphatase activities (Table 1). Particulate InsP₃ phosphatase activity showed regional variations, with the highest levels in the cerebellum, almost triple that of the other regions; the midbrain, brain stem, corpus striatum, cerebral cortex and hippocampus displayed one-third or slightly less than one-third of the cerebellar levels. The soluble phosphatase, whose activity was much lower than that of the particulate enzyme (Table 2), showed a similar regional distribution, with highest levels in the cerebellum (results not shown).

Membrane-associated InsP₃ kinase activity displayed a different regional pattern from phosphatase activity, with highest levels in the hippocampus and corpus striatum, areas which had lower levels of InsP₃ phosphatase. Cerebellum activity was 84.2% of hippocampal levels; midbrain displayed levels only 15.3% of those of hippocampus, and activity was lowest in the brain stem. The soluble kinase, whose specific activity was about 3 times that of the particulate enzyme (Table 2), nevertheless showed a similar regional distribution (results not shown).

Soluble versus particulate localization of InsP₃ and InsP₄ binding, InsP₃ kinase and InsP₃ phosphatase

Previous studies have shown that InsP₃ and InsP₄ binding in brain is predominantly membrane-associated [8,35]. In several tissues, a soluble InsP₃ 3-kinase has been characterized and

Table 2. Detergent extraction of InsP_3 phosphatase, InsP_3 kinase, InsP_4 binding and InsP_3 binding from rat cerebellar membranes

Salt-washed membranes and 100 000 *g* supernatant fractions were prepared as described in the Materials and methods section. Detergent-extracted membranes were resuspended in buffer in the absence of detergent. The protein from three cerebella recovered in each fraction was as follows: supernatant 120 mg, membranes 100 mg, solubilized membranes 65 mg and extracted membranes 35 mg. InsP_3 kinase was assayed from 1 to 5 min at 24 °C with 3–10 μg of protein in 400 μl . InsP_3 phosphatase was assayed from 0.5 to 3 min with 2–10 μg of protein in 400 μl . Both enzymes were assayed with 50 nM- InsP_3 . InsP_4 and InsP_3 binding was determined as described in the Materials and methods section. For all measurements, 100 % activity recovered was the supernatant plus membrane activities. Assays were conducted in duplicate. Data are means $\pm$ S.E.M. for five independent determinations.

Fraction	Activity (pmol/min per mg) [% of activity recovered]		Activity (pmol/mg) [% of activity recovered]	
	InsP_3 phosphatase	InsP_3 kinase	InsP_4 binding	InsP_3 binding
100 000 <i>g</i> supernatant	76 $\pm$ 5 [9.2]	98 $\pm$ 14 [66.9]	0.02 $\pm$ 0.01 [2.9]	0.21 $\pm$ 0.06 [3.5]
Salt-washed membranes	893 $\pm$ 76 [90.8]	58 $\pm$ 17 [33.1]	0.68 $\pm$ 0.35 [97.1]	6.9 $\pm$ 1.1 [96.5]
1 % -CHAPS-solubilized membranes, supernatant	2435 $\pm$ 330 [161.0]	37.2 $\pm$ 12.5 [13.8]	0.42 $\pm$ 0.12 [27.3]	2.7 $\pm$ 0.75 [24.5]
1 % -CHAPS-extracted membranes	286 $\pm$ 77 [10.2]	27.7 $\pm$ 5.4 [5.5]	0.20 $\pm$ 0.10 [10.3]	2.6 $\pm$ 1.2 [12.7]

purified, although several particulate kinase activities have been demonstrated [23–29]. Both soluble and particulate forms of InsP_3 5-phosphatase have been described and purified [14–22]. In rat cerebellum we have confirmed the predominantly particulate association of InsP_3 and InsP_4 binding (Table 2). In contrast, most of the InsP_3 kinase activity was soluble, though salt-washed cerebellar membranes displayed approx. 30–50 % as much total InsP_3 kinase activity as the soluble fraction. The K_m for both soluble and membrane-associated kinases was 250 nM and the V_{max} values were 670 and 300 pmol/min per mg respectively. Over 90 % of the InsP_3 phosphatase was particulate ($K_m = 10 \mu\text{M}$, $V_{\text{max}} = 250 \text{ nmol/min per mg}$), with the total soluble portion exhibiting less than 10 % of the activity of salt-washed membranes. These values, in addition to its ability to hydrolyse InsP_4 to $\text{Ins}(1,3,4)\text{P}_3$, but at a slower rate, are consistent with data reported by others in brain and peripheral tissues.

All four activities, the two inositol phosphate-binding activities and the two particulate enzymes, could be solubilized by the addition of 1 % CHAPS. Most of the activity appeared to be solubilized, since only 5–13 % of the original activities were recovered in the detergent-extracted membrane fraction. However, for InsP_3 and InsP_4 binding and the InsP_3 kinase, all of the activity was not recovered in the detergent extract, probably, for InsP_3 and InsP_4 binding, because the detergent markedly inhibits ligand binding. This inhibition is apparently reversible, since dilution of detergent-solubilized receptor and reconstitution into lipid vesicles resulted in a tripling of [^3H] InsP_3 and [^3H] InsP_4 binding (C. D. Ferris & A. B. Thiebert, unpublished work). The reasons for the limited recovery of kinase in the detergent-solubilized preparation are less clear. One factor may be an irreversible loss of activity during the solubilization. InsP_3 kinase activity is reportedly labile during purification [28]. As with the binding, the other major factor may be detergent inhibition of enzyme activity. In preliminary experiments, increasing CHAPS concentrations from 0.1 % to 1 % in the assay potentially inhibited InsP_3 kinase activity (A. Thiebert, unpublished work).

In contrast with the inhibition of binding and kinase activity, InsP_3 phosphatase activity was significantly enhanced in detergent-solubilized fractions. This may result from removal of inhibitors which diminish enzymic activity in the membranes. Alternatively, the detergent may activate the enzyme or allow better access to the substrate.

Partial purification and separation of InsP_3 and InsP_4 binding and InsP_3 kinase and InsP_3 phosphatase activities

In confirmation of our previous findings [12], InsP_3 -binding activity adhered to a heparin-agarose resin and could be eluted with a high-ionic-strength buffer. In addition to InsP_3 binding, InsP_4 -binding activity and InsP_3 kinase similarly adhered to and were eluted from the heparin-agarose resin under the same conditions (Table 3). In contrast, 95 % of the InsP_3 phosphatase activity did not interact with this resin. The InsP_3 phosphatase therefore could not represent the major source of InsP_3 - or InsP_4 -binding activity. As previously published [12], InsP_3 -binding activity adhered to the lectin Con A-Sepharose, and could be specifically eluted with α -methyl mannoside. Most of the InsP_3 kinase and InsP_4 -binding activity, on the other hand, did not adhere to this resin, and so both could be fully resolved from InsP_3 -binding activity (Table 3).

Another affinity column was used to resolve the InsP_3 3-kinase and InsP_4 -binding site. InsP_4 binding was not affected by 10 μg of calmodulin/ml. However, the stimulation and interaction of the soluble InsP_3 kinase with calmodulin has been documented in several systems and used as a purification step [28]. Over 90 % of the added detergent-solubilized InsP_3 kinase activity adhered to a calmodulin affinity column in the presence of Ca^{2+} , and approx. 50 % could be eluted from the resin by EGTA. In contrast, most of the InsP_4 -binding activity did not adhere to the calmodulin column; of the activity recovered, 90 % was recovered in the flow-through and only 10 % was recovered in the eluate and so could be resolved from the InsP_3 kinase.

Inositol phosphate specificity of partially purified InsP_3 and InsP_4 receptor proteins

In our characterization of membrane-associated InsP_4 binding [35], InsP_3 and InsP_4 had similar potencies at the InsP_4 -binding site, with K_i values of approx. 300 nM. The inositol phosphate specificity of the detergent-solubilized InsP_4 -binding activity in the eluate of the heparin-agarose columns was remarkably different (Table 4). This partially purified preparation displayed very high affinity and selectivity for InsP_4 over InsP_3 , with IC_{50} values of 10 nM for InsP_4 and $> 300 \text{ nM}$ for InsP_3 (Table 4). The partially purified InsP_3 receptor protein possessed more than 100 times greater affinity for InsP_3 than for InsP_4 . InsP_6 was approx.

Table 3. Resolution of detergent-solubilized InsP_3 phosphatase, InsP_3 kinase, InsP_3 -binding and InsP_4 -binding activities from rat cerebellum

Enzyme and binding assays were performed as described in Table 2 legend and the Materials and methods section. Protein recovery was as follows: CHAPS-solubilized 65 mg, heparin-agarose flow-through 50 mg, heparin-agarose eluate 6 mg, Con A-Sepharose flow-through 2.5 mg, Con A-Sepharose eluate 0.4 mg, calmodulin-agarose flow-through 1.8 mg, calmodulin-agarose eluate 0.45 mg. For all measurements, 100% activity recovered was that present in the solubilized membrane fraction. Assays were conducted in duplicate. Data are means $\pm$ S.E.M. for five independent experiments (nd, not determined).

Fraction	Sp. activity (pmol/min per mg) [% of activity recovered]		Sp. activity (pmol/mg) [% of activity recovered]	
	InsP_3 phosphatase	InsP_3 kinase	InsP_4 binding	InsP_3 binding
1%-CHAPS-solubilized	2435 $\pm$ 330 [100]	37.2 $\pm$ 12.5 [100]	0.42 $\pm$ 0.12 [100]	2.7 $\pm$ 0.75 [100]
Heparin-agarose flow-through	2799 $\pm$ 450 [88.4]	0.67 $\pm$ 0.42 [1.4]	0.018 $\pm$ 0.006 [3.3]	0.034 $\pm$ 0.02 [1.0]
Heparin-agarose 0.5 M-NaCl eluate	940 $\pm$ 200 [3.6]	121 $\pm$ 20 [30.1]	2.9 $\pm$ 1.3 [63.7]	13.7 $\pm$ 3.2 [47.0]
Con A-Sepharose flow-through	2156 $\pm$ 307 [3.4]	231 $\pm$ 28 [23.9]	3.2 $\pm$ 1.1 [29.3]	0.3 $\pm$ 0.1 [0.4]
Con A-Sepharose α -methyl mannoside eluate	< 2 [< 0.01]	51 $\pm$ 8 [1]	1.5 $\pm$ 0.4 [2.2]	109 $\pm$ 18 [24.9]
Calmodulin-agarose flow-through	nd	20 $\pm$ 3 [1.5]	2.6 $\pm$ 0.5 [17.1]	nd
Calmodulin-agarose EGTA eluate	nd	655 $\pm$ 15 [12.1]	1.0 $\pm$ 0.1 [1.6]	nd

Table 4. Inositol phosphate specificity of purified InsP_3 and partially purified InsP_4 receptors

Binding was performed as described in the Materials and methods section. Membranes (50 μ g) were incubated with 3 nM-[^3H] InsP_4 or [^3H] InsP_3 and the indicated unlabelled inositol phosphates for 10 min at 0 $^\circ\text{C}$. Specific binding was determined by the spun-column method. Non-specific binding was determined with 10 μM unlabelled InsP_3 or InsP_4 and was approx. 5–10% of totals. Assays were performed in triplicate. Data are mean values of three independent determinations which varied by less than 10% (nd, not determined). IC_{50} , concn. of ligand giving 50% inhibition of binding.

	Affinity at receptor (IC_{50} , nM)	
	InsP_3	InsP_4
$\text{Ins}(1,4,5)\text{P}_3$	10	> 300
$\text{Ins}(1,3,4)\text{P}_3$	> 1000	nd
$\text{Ins}(2,4,5)\text{P}_3$	500	> 300
$\text{Ins}(1,3,4,5)\text{P}_4$	> 1000	10
$\text{Ins}(1,4,5,6)\text{P}_4$	nd	300
InsP_6	> 1000	50

4–5-fold weaker than InsP_4 at displacing InsP_4 binding, although the purity of this InsP_6 preparation was not determined. Another InsP_4 isomer, $\text{Ins}(1,4,5,6)\text{P}_4$, was 30-fold weaker than $\text{Ins}(1,3,4,5)\text{P}_4$.

DISCUSSION

The main findings of this study are the solubilization of the InsP_4 -binding site, the demonstration of a significant particulate InsP_3 kinase in brain, and the physical separation of InsP_3 - and InsP_4 -binding proteins from each other and from the InsP_3 kinase and InsP_3 phosphatase. The InsP_3 and InsP_4 binding and InsP_3 kinase activities adhere to heparin-agarose resins, but are

separated by a Con A-Sepharose resin, to which InsP_3 -binding activity adheres but InsP_4 binding and InsP_3 kinase activity flow through. Neither of the inositol phosphate-binding activities is likely to involve the InsP_3 phosphatase, since 95% of the phosphatase does not adhere to the heparin-agarose resin. Both the InsP_3 kinase and InsP_4 -binding activity adhere to heparin-agarose, but not to Con A-Sepharose. However, these two activities can be separated, since the InsP_3 kinase adheres specifically to a calmodulin affinity column to which most of the InsP_4 -binding activity does not adhere.

In rat brain tissue we have found InsP_3 5-phosphatase activity to be predominantly particulate, consistent with results of others in brain and other tissues. Among different brain regions, InsP_3 phosphatase activity is highest in the cerebellum, with other regions displaying activity one-third of cerebellar levels. This high concentration within the cerebellum resembles the localization of InsP_3 -receptor-binding sites [7,8], as well as other elements of the phosphoinositide cycle such as phospholipase C and protein kinase C [39]. This pattern of regional localization supports a physiological role for this enzyme in terminating the second-messenger activities of InsP_3 .

Most studies of InsP_3 kinase have reported activity to be predominantly soluble, especially in brain tissue. In rat cerebellum we find that salt-washed membranes have about one-third to one-half the activity of soluble fractions. The regional distribution of InsP_3 kinase activity differs from the InsP_3 phosphatase activity and InsP_3 binding, but closely resembles InsP_4 binding.

Finally, we have demonstrated the solubilization and partial purification of the InsP_4 -binding site. Our finding that InsP_4 -binding activity cannot be explained by interactions with InsP_3 phosphatase or InsP_3 kinase, or by binding to the InsP_3 receptor, suggests that the InsP_4 binding site represents a physiologically relevant receptor. Additional evidence in support of this comes from the inositol phosphate specificity of the partially purified InsP_4 -binding protein, which displayed high affinity and selectivity for InsP_4 in contrast with other inositol phosphates. In

the absence of a clearly defined physiological role for InsP_4 , one cannot compare the inositol phosphate specificity of the binding site with that of InsP_4 physiological effects. The inositol phosphate specificity of the purified InsP_4 -binding protein differs markedly from the particulate InsP_4 -binding activity [35], whose functional significance is unclear. The shallow displacement curves for various inositol phosphates at the particulate InsP_4 -binding sites suggest that it represents a mixture of sites, with no more than a small percentage reflecting the high-affinity InsP_4 -binding site of partially purified soluble preparations. Significant insight into the function of the InsP_3 -binding protein has come recently from the reconstitution of InsP_3 -induced Ca^{2+} release from lipid vesicles into which the purified InsP_3 -binding protein has been inserted [13]. A similar approach may help to elucidate the role of InsP_4 as a second messenger in the cell.

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