Inositol 1,3,4,5-tetrakisphosphate and inositol hexakisphosphate receptor proteins: Isolation and characterization from rat brain

(inositol phosphates/cyclic AMP-dependent protein kinase/protein kinase C)

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ABSTRACT High-affinity, membrane-associated inositol 1,3,4,5-tetrakisphosphate (IP₄) and inositol hexakisphosphate (IP₆) binding proteins were solubilized and isolated utilizing a heparin-agarose resin followed by an IP₄ affinity resin. The IP₄ receptor comprises a protein complex of 115-, 105-, and 50-kDa subunits, all of which comigrate under native conditions. The Kᵣ of the receptor for IP₄ is 12 nM, whereas inositol 1,3,4,5,6-pentakisphosphate (IP₅), IP₆, and inositol 1,4,5-trisphosphate (IP₃) are 50%, 30%, and 15%, respectively, as potent. Two protein complexes copurify with the IP₄ receptor fraction. A 182/123-kDa complex elutes first from the affinity column followed by a 174/84-kDa protein complex, which elutes at higher salt. Both complexes show high affinity for IP₄ (Kᵣ = 3–4 nM). IP₅, IP₆, and IP₃ display approximately 25%, 10%, and 0.1%, respectively, the affinity of IP₄. Ligand binding to IP₄ and IP₆ receptors is inhibited 50% by heparin at 0.1 μg/ml. IP₄ receptor proteins are stoichiometrically phosphorylated by cyclic AMP-dependent protein kinase and protein kinase C, whereas negligible phosphorylation is observed for the IP₆ receptor.

Several inositol polyphosphates appear to be biological messenger molecules. Inositol 1,4,5-trisphosphate (IP₃) releases calcium from nonmitochondrial stores (1) by binding to a receptor protein, which has been isolated (2), shown by functional reconstitution to contain the IP₃ recognition site and its associated calcium channel (3), localized by immunohistochemistry to subdivisions of the endoplasmic reticulum (4), and molecularly cloned from mouse brain (5) and from rat brain (6, 7). Inositol 1,3,4,5-tetrakisphosphate (IP₄) is formed in mammalian tissues by selective phosphorylation of IP₃ by a 3-kinase (9–14). The biological function of IP₄ is less clear than that of IP₃, but IP₄ may participate in the movement of calcium into the cell and/or in maintaining levels of the IP₃-sensitive calcium pools (15–18). More recently, evidence has accumulated favoring a biological role for inositol 1,3,4,5,6-pentakisphosphate (IP₅) and inositol hexakisphosphate (IP₆). IP₅ and IP₆ occur in mammalian tissues in substantial levels (19–22), can influence calcium flux (23), and alter electrophysiological and cardiovascular events (24).

The physiological role of IP₄ has been clarified by purification of its receptor protein, employing conventional techniques (2) or, more recently, IP₄ affinity chromatography (25). Similar strategies might clarify functions of IP₅ and IP₆. We (26, 27) and others (28–31) have identified [³H]IP₄ binding sites associated with putative receptor proteins. [³H]IP₆ binding to brain membranes has also been characterized (32, 33). In the present study we have used heparin and IP₄ affinity chromatography to purify and characterize IP₃ and IP₄ receptor proteins. In addition, we demonstrate that IP₄ receptors are phosphorylated in vitro by cyclic AMP-dependent protein kinase A (PKA) and protein kinase C (PKC).

MATERIALS AND METHODS

Materials. [³H]IP₄ (17 Ci/mmol; 1 Ci = 37 GBq), [³H]IP₆ (12 Ci/mmol), and Formula 963 scintillation cocktail were obtained from DuPont/NEN. All unlabeled inositol phosphates were obtained from Calbiochem. Heparin, heparin-agarose, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), bovine gamma globulin, and polyethylene glycol no. P-3640 (M₉, 3350) were obtained from Sigma. Concanaevalin A (Con A)-Sepharose was purchased from Pharmacia. Other reagents were from Sigma.

Preparation of Membranes and Membrane Solubilization.

Rat cerebellar membranes were prepared as described (26, 27). Briefly, cerebella from 30 male Sprague–Dawley rats (200–300 g) were homogenized (Polytron setting 6, 8 s) at 0°C in 250 ml of homogenization buffer [50 mM Tris-HCl (pH 7.7), containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 25 mg of phenylmethylanesulfonyl fluoride, 1.2 mg of chymostatin, 1.2 mg of antipain, 1.2 mg of pepstatin, 2.4 mg of aprotinin, 2.4 mg of leupeptin, and 62.5 mg of N-carbobenzoxyphenylalanine]. Homogenates were centrifuged (15 min, 45,000 × g), and membrane pellets were resuspended in 250 ml of homogenization buffer. Membrane proteins were solubilized with 1% CHAPS for 30–60 min and centrifuged (30–45 min, 45,000 × g).

Binding Assays for IP₄ and IP₆. Membrane binding of [³H]IP₄ or [³H]IP₆ was determined by incubating 50 μg of membranes with 0.030 μCi of [³H]IP₄ or [³H]IP₆ in 400 μl of 25 mM Tris-HCl (pH 8.0) with 1 mM EDTA for 10 min at 0°C. Bound ligand was separated from unbound by centrifugation for 10 min at 12,000 × g. Supernatants were aspirated and pellets were solubilized with 100 μl of 1% SDS and transferred to minivials; 4–5 ml of Formula 963 scintillation cocktail was added and radioactivity was determined. Non-specific binding was determined by including 3 μM IP₄ or IP₆ in the assay.

Binding to detergent-solubilized fractions was determined by a polyethylene glycol precipitation procedure. Solubilized fractions (10–100 μl) were incubated with 0.03 μCi of [³H]IP₄ or [³H]IP₆ in a total volume of 400 μl of 25 mM Tris-HCl (pH 7.4 or 8.0) plus 1 mM EDTA for 10 min at 0°C. Receptor–ligand complex was precipitated by addition of 100 μl of 2.4 M ammonium sulfate to the assay, with stirring on a shaking wheel.

Abbreviations: PKA, cyclic AMP-dependent protein kinase; IP₃, inositol 1,4,5-trisphosphate; IP₄, inositol 1,3,4,5-tetakisphosphate; IP₅, inositol hexakisphosphate; IP₆, inositol 1,3,4,5,6-pentakisphosphate; PKC, protein kinase C; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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bovine gamma globulin (5.0 mg/ml) as a carrier protein and then 1.0 ml of 25% polyethylene glycol (M, 3350) for 10 min at 0°C and centrifuged for 10–15 min at 12,000 x g. Supernatants were aspirated and pellets were solubilized as described for membranes. Nonspecific binding was determined by including 3 μM unlabeled IP₄ or IP₃ in the assays.

Synthesis of IP₄ Affinity Resin by Coupling of 1-O-(3-Aminopropyl-1-Phospho)-myo-Inositol 3,4,5-Trisphosphate to Affi-Gel 10. Affi-Gel 10 (Bio-Rad) resin (25 ml, corresponding to ca. 0.25 mmol of active ester sites) was prewashed with water at 4°C and stirred at this temperature with a precooled solution of 106 mg (0.152 mmol) of 1-O-(3-aminopropyl-1-phospho)-myo-inositol 3,4,5-trisphosphate (see Fig. 1 and ref. 38) dissolved in 20 ml of water containing 170 mg of NaHCO₃ (pH 8.5). After stirring at 4°C for 1.5 hr and overnight at 12°C, the IP₄ affinity resin was isolated by filtration on a sintered glass funnel, washed with 100 ml of 4°C water, and stored at this temperature as an aqueous suspension. Unreacted active ester groups were hydrolyzed by stirring overnight at pH 8.5 or by capping with ethanolamine.

Chromatographic Separations of Binding Activities. Solubilized membranes (250 ml) were adjusted to 250 mM NaCl, incubated with 12.5 ml of packed, washed heparin-agarose for 15 min on a rotator, and poured into a column, and nonadherent proteins were collected and discarded. The column was washed with 150 ml of homogenization buffer containing 1% CHAPS and 250 mM NaCl and eluted with 50 ml of homogenization buffer containing 1% CHAPS and 750 mM NaCl for 20 min. The eluate was collected, the resin was washed with an additional 15 ml of elution buffer, and the two eluates were pooled.

The heparin eluate was adjusted to 1 mM MgCl₂ and 1 mM CaCl₂, incubated with 4.5 ml of packed Con A-Sepharose for 45 min on a rotator, and poured into a column, the nonadherent protein fraction was collected, and EDTA was added to a final concentration of 5 mM. Approximately 80–90% of the 260-kDa IP₄ receptor adhered to the Con A column. The column was washed with 20 ml of homogenization buffer containing 1% CHAPS, 1 mM MgCl₂, 1 mM CaCl₂, and 750 mM NaCl. The flow-through and wash were pooled and concentrated using Amicon Centriprep-30 concentrators. The 60 ml of flow-through and wash was concentrated 10-fold to 6 ml final volume.

The concentrated Con A flow-through plus wash was diluted with 44 ml of 50 mM Tris 7.4/1 mM EDTA/1% CHAPS plus half the concentration of the protease inhibitors of homogenization buffer. This fraction was loaded onto an IP₄ column adapted to the FPLC (dimensions, 10 cm x 3 cm) at a rate of 0.2 ml/min. The column was washed with 10 ml of 150 mM NaCl in the above buffer, and the binding activities were eluted with a gradient of 0.15 M to 1.5 M NaCl at 0.2 ml/min in a volume of 50 ml. Thirty 1.5-ml fractions were collected and analyzed for binding activity (30 μl) and for proteins (100 μl) by SDS/PAGE.

Phosphorylation by PKC and PKA. Receptor phosphorylation was determined by incubating IP₄ affinity column fractions with 10 mM MgCl₂, 50 mM ATP, and 5 μg of purified PKC or PKA per ml as described (8, 34, 35). For PKC phosphorylation, 1 mM CaCl₂, 5 μg of diacylglycerol per ml, and 30 μg of phosphatidylserine were included as described (36). Reactions were carried out at 30°C. No phosphorylation was detected in the absence of added Ca²⁺. After various times SDS sample buffer was added to stop phosphorylation and samples were separated by SDS/PAGE.

RESULTS

Purification of IP₃ and IP₄ Receptor Binding Proteins. Previously we observed [³H]IP₃ binding to brain membranes and to solubilized and partially purified preparations (26, 27).

Similar to the IP₃ receptor, the IP₄ receptor adheres to a heparin-agarose resin but, unlike the IP₃ receptor, IP₄ binding activity does not adsorb to Con A-Sepharose, permitting separation of the two receptors.

The binding specificity and affinity of [³H]IP₄ to the solubilized preparation differ from the membrane preparation (26, 27); thus [³H]IP₄ may bind to multiple sites in the membrane, or the IP₄ receptor may be allosterically coupled to other proteins in the membrane-bound state. Therefore, it is difficult to quantify the recovery of specific IP₄ receptors in the solubilization process. Of the total [³H]IP₄ binding activity in rat cerebellar membranes, 60% appears to be recovered in the supernatant following solubilization with 1% CHAPS for 45 min at 4°C (Table 1). This procedure also solubilizes [³H]IP₃ binding activity. The inositol phosphate specificity of solubilized [³H]IP₄ binding sites resembles that of intact membranes (33). IP₄ displays a K₅ of about 12 nM, with IP₄ and IP₃ being 2- to 3-fold less potent. IP₃ has only about 5% of the affinity of IP₄ for the binding sites. Similar to [³H]IP₃ binding, ≈62% of [³H]IP₄ binding is recovered in the solubilization process (Table 1).

[³H]IP₄ and [³H]IP₃ binding activities adhere to a heparin-agarose resin and can be specifically eluted with 0.75 M NaCl, providing a 7- to 8-fold purification of IP₄ and IP₃ binding activities with slightly more than 50% recovery of activity (Table 1). IP₃ and IP₄ binding activities flow through the Con A-Sepharose resin, which retains the IP₄ binding activity and provides an additional 3-fold increase in specific activity for IP₄ and IP₃ binding.

IP₄ and IP₃ binding activities have been separated using a P-1 tethered phosphodiester of IP₄ (ref. 38) coupled through a three-carbon spacer to Affi-Gel 10 (Fig. 1). This provides an excellent purification step since >90% of the protein applied in the IP₄ column does not interact with the resin. Proteins that specifically adhere to the resin are eluted by a NaCl gradient (Fig. 2; Table 1). A single peak of [³H]IP₄ binding elutes first with ~400 mM NaCl, followed by two successive, overlapping peaks enriched in [³H]IP₄ binding, which elute between 600 and 900 mM NaCl (Fig. 2).

The inositol phosphate specificity of the first eluted peak is similar to [³H]IP₃ binding in membranes and in crude solubilized preparations (Fig. 3). The K₅ for IP₃ is 12 nM, with IP₄, IP₃, and IP₄ displaying approximately 50%, 30%, and 5%, respectively, of the potency of IP₃. By contrast, the second and third peaks display a high affinity (K₅ = 3–4 nM) and selectivity for IP₄. IP₄ is approximately 4, 10, and 1000 times more potent than IP₃, IP₆, and IP₃, respectively (Fig. 3).

The IP₄ affinity column provides a striking enrichment in IP₄ and IP₃ binding activities. The specific activity for IP₄ binding in this column is increased 20-fold over the Con A flow-through, providing a 300-fold purification over the crude detergent-solubilized preparation (Table 1). Both peaks of IP₄ binding are purified 100-fold compared with the Con A

Table 1. Purification of IP₃ and IP₄ receptors by affinity chromatography

<table>
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<th>Purification step</th>
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Inositol phosphate binding activities were assayed by polyethylene glycol precipitation with 0.03 μC of [³H]IP₃ or [³H]IP₄ at pH 7.4. Binding (B_max) is expressed as pmol/mg of protein. A single experiment, representative of four independent purifications, is depicted.
Flow-through and 2300-fold compared with the detergent-solubilized preparation.

To ascertain the identity of these binding sites separated by affinity chromatography, we conducted SDS/PAGE analysis (Fig. 2). The IP₆ receptor peak displays two doublets at approximately 115 and 105 kDa and a singlet at 50 kDa. To determine whether these are separate, unrelated proteins or parts of a complex of subunits comprising the IP₆ receptor, we conducted size exclusion chromatography with an FPLC Superose 6 column. A single peak of [³H]IP₆ binding is obtained, corresponding to an estimated molecular size of 300–350 kDa in the presence of detergent and containing all three bands (data not shown). This protein complex also

![Figure 1](image1.png)

**Fig. 1.** Synthesis of IP₄ affinity resin by coupling of 1-O-(3-aminopropyl-1-phospho)-myo-inositol 3,4,5-trisphosphate to Affigel 10.

![Figure 2](image2.png)

**Fig. 2.** (A) Elution profile of IP₆ and IP₄ receptors from the IP₆ affinity resin. Con A flow-through plus wash from 30 rat cerebella were chromatographed on an aminopropyl IP₄ Affi-Gel 10 column adapted to FPLC and eluted by a 0.15–1.5 M NaCl gradient in 1.5-ml fractions. Due to interference of detergent in protein assays, protein concentration was determined by comparison to bovine serum albumin staining on SDS/PAGE. Binding of [³H]IP₄ and [³H]IP₆ was assayed by polyethylene glycol precipitation using 0.03 μCi of [³H]IP₄ (○) or [³H]IP₆ (■) radiolabeled ligand and 50 μl of each column fraction. Nonspecific binding was determined with 3 μM unlabeled IP₄ or IP₆ and was <10% of total binding. A typical elution profile of five independent chromatographic runs is depicted. (B) SDS/PAGE analysis of IP₄ affinity column fractions. Aliquots (100 μl) of 1.5-ml fractions were analyzed on 7.5% gels. Lanes are denoted as they correspond to the column fractions. Similar gel profiles were observed in five independent column separations. Sizes are indicated in kDa.

![Figure 3](image3.png)

**Fig. 3.** Inositol phosphate specificity of IP₆ and IP₄ receptors. (A) Peak 1 from IP₄ affinity column eluting at 400 mM NaCl. (B) Peak 2 eluting at 750 mM NaCl. (C) Peak 3 eluting at 900 mM NaCl. Binding was assayed by polyethylene glycol precipitation using 0.03 μCi of [³H]IP₄ and 30 μl of each fraction at pH 7.4. Data are means from three independent experiments performed in duplicate with standard errors of <10%. ○, IP₃; ■, IP₄; □, IP₃; ●, IP₆.
cannot be resolved by cation- or anion-exchange chromatography. Together with the finding that the three bands emerge simultaneously from the IP₄ affinity chromatography column, we conclude that all three are components of the IP₄ receptor binding protein complex.

The IP₄ receptor peak that elutes first from the IP₄ affinity column (peak 2) displays a single intensely stained protein band at 182 kDa, with a less pronounced protein observed at 123 kDa (Fig. 2). The second IP₄ receptor binding (peak 3) is also comprised of two protein bands, one of which migrates at 84 kDa and a second, less intense band, which migrates at 174 kDa.

**Properties and Phosphorylation of Purified IP₄ and IP₆ Receptor Proteins.** IP₄ and IP₆ receptor binding activities from the IP₄ affinity column are reduced 25–40% by warming to 30°C for 15 min and abolished by heating to 90°C for 10 min. Thermolysin (100 μg/ml) abolishes binding activity (data not shown). The pH optimum for IP₄ binding is 6.0–6.5. The two IP₄ binding fractions have pH optima of 5.0. Heparin potently inhibits IP₄ and IP₆ receptor binding (IC₅₀ = 0.1 μg/ml). Ca²⁺ and Mg²⁺ enhance IP₄ and IP₆ binding to purified receptors 20–50% at 0.1 mM and inhibit binding 25–75% at 1 mM (data not shown).

To determine whether [³H]IP₄ and [³H]IP₆ binding might involve enzymes associated with inositol phosphate phosphorylation or dephosphorylation, we assayed each of the peaks from the IP₄ affinity column for phosphatase and kinase activity utilizing [³H]IP₃ and [³H]IP₆ as substrates. None of the three protein peaks displays any enrichment of these enzymatic activities (data not shown).

Like the IP₃ receptor (35), the IP₄ receptors (182-, 123-, and 84-kDa proteins) are phosphorylated by PKA and PKC (Fig. 4). Phosphorylation is rapid, reaching a plateau by 10 min at 30°C for the 182-kDa protein, with 1.2 mol of phosphate incorporated per mol of receptor. PKC phosphorylation of the 182-kDa IP₄ receptor plateaus by 20 min, with 1.0 mol per mol of receptor incorporated. The 123-kDa protein also incorporates nearly 1.0 mol per mol of phosphate with PKC but only 0.4 mol per mol with PKA. The 84-kDa protein is phosphorylated with kinetics similar to the 182-kDa protein but incorporates 0.4 mol per mol with PKA and 0.5 mol per mol with PKC. No phosphorylation of the 174-kDa protein was detected. In addition, very limited IP₆ receptor phosphorylation is observed. The lower band of the 115-kDa doublet is phosphorylated by PKA, but <0.1 mol of phosphate per mol of receptor is incorporated (data not shown).

**DISCUSSION**

The inositol phosphate specificity of ligand binding to the purified proteins and their lack of enriched phosphatase and kinase activities indicate that we have isolated putative IP₄ and IP₆ receptor proteins. IP₃ has substantial affinity for the isolated binding proteins. Thus, these proteins might include physiological IP₃ receptors.

Successful purification employed an IP₄ affinity chromatography column that has substantial affinity for the IP₄ and IP₆ receptors. The utility of inositol phosphate affinity chromatography is also evident from our purification of IP₃ receptors with an IP₃ Affi-Gel affinity column (25).

The inositol phosphate specificity of the purified IP₄ receptor resembles the pattern of [³H]IP₆ binding to brain membranes (33). This IP₆ binding site has distinct localizations in the brain, consistent with a neuronal, synaptic role (24); however, we have not yet determined if these receptors are also present in nonneuronal tissues. Whether the purified IP₆ receptor protein physiologically interacts primarily with IP₄ or IP₆ is unclear, though it possesses about a 2-fold higher affinity for IP₆ than IP₄.

Nicoletti et al. (32) reported high affinity binding of [³H]IP₆ in brain and pituitary membranes, which differs from the receptor we have purified. For instance, Nicoletti et al. (32) found IP₆ only 0.5% as potent as IP₄, whereas we observe only a 2-fold lower potency of IP₆. Moreover, the Bₘₐₓ and temperature dependence differ markedly for the two IP₆ binding sites.

We have isolated IP₄ receptor proteins of molecular sizes 182, 123, 174, and 84 kDa. These proteins display similar high
affinity for IP₃ (Kᵦ = 3–4 nM) and inositol phosphate selectivity, suggesting that they might comprise a single protein complex, though protein cleavage may have occurred during purification. Subtle differences in the inositol phosphate specificity may be a result of post-translational modification of the IP₃ receptor proteins. Further characterization will determine the relationship of these protein subunits. Donie et al. (29) partially purified [³H]IP₃ binding activity from porcine cerebellar membranes using heparin-agarose. Their binding site displays low nanomolar affinity for IP₃ with a somewhat different inositol phosphate specificity but a similar pH optimum. IP₃ binding sites reported in peripheral tissues display inositol phosphate specificity and receptor density quite different from the receptor we have isolated (30–31).

Isolation of the IP₃ and IP₆ receptor proteins may clarify physiological roles of these inositol phosphates. Both have been reported to influence calcium disposition. IP₆ acts synergistically with IP₃ in regulating calcium release (17, 18) and IP₆ affects calcium accumulation in cultured brain cells (23). With IP₃ receptor protein reconstituted into lipid vesicles we demonstrated IP₃ stimulation of calcium flux (3). IP₃ inhibits [³H]IP₃ binding potently in membranes. Following solubilization, though, this inhibition decreases dramatically, suggesting that IP₃ and IP₆ receptors interact allosterically, as has been recently proposed (36, 37). Similar reconstitution experiments for IP₃ and IP₆ receptors possibly together with IP₃ receptors may identify their roles in calcium regulation or in other cellular or extracellular actions.

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