Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 3165–3169, April 1991
Neurobiology

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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Contributed by Solomon H. Snyder, January 4, 1991

ABSTRACT **High-affinity, membrane-associated inositol 1,3,4,5-tetrakisphosphate (IP_4) and inositol hexakisphosphate (IP_6) binding proteins were solubilized and isolated utilizing a heparin-agarose resin followed by an IP_4 affinity resin. The IP_6 receptor comprises a protein complex of 115-, 105-, and 50-kDa subunits, all of which comigrate under native conditions. The K_d of the receptor for IP_6 is 12 nM, whereas inositol 1,3,4,5,6-pentakisphosphate (IP_5), IP_4, and inositol 1,4,5-trisphosphate (IP_3) are 50%, 30%, and 15%, respectively, as potent. Two protein complexes copurify with the IP_4 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 IP_4 (K_d = 3–4 nM). IP_5, IP_6, and IP_3 display approximately 25%, 10%, and 0.1%, respectively, the affinity of IP_4. Ligand binding to IP_6 and IP_4 receptors is inhibited 50% by heparin at 0.1 μg/ml. IP_4 receptor proteins are stoichiometrically phosphorylated by cyclic AMP-dependent protein kinase and protein kinase C, whereas negligible phosphorylation is observed for the IP_6 receptor.**

Several inositol polyphosphates appear to be biological messenger molecules. Inositol 1,4,5-trisphosphate (IP_3) releases calcium from nonmitochondrial stores (1) by binding to a receptor protein, which has been isolated (2), shown by functional reconstitution to contain the IP_3 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 (IP_4) is formed in mammalian tissues by selective phosphorylation of IP_3 by a 3-kinase (9–14). The biological function of IP_4 is less clear than that of IP_3, but IP_4 may participate in the movement of calcium into the cell and/or in maintaining levels of the IP_3-sensitive calcium pools (15–18). More recently, evidence has accumulated favoring a biological role for inositol 1,3,4,5,6-pentakisphosphate (IP_5) and inositol hexakisphosphate (IP_6). IP_5 and IP_6 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 IP_3 has been clarified by purification of its receptor protein, employing conventional techniques (2) or, more recently, IP_3 affinity chromatography (25). Similar strategies might clarify functions of IP_4 and IP_6. We (26, 27) and others (28–31) have identified [^{3}H]IP_4 binding sites associated with putative receptor proteins. [^{3}H]IP_6 binding to brain membranes has also been characterized (32, 33). In the present study we have used heparin and IP_4 affinity chromatography to purify and characterize IP_4 and IP_6 receptor proteins. In addition, we demonstrate that IP_4 receptors are phosphorylated *in vitro* by cyclic AMP-dependent protein kinase A (PKA) and protein kinase C (PKC).

MATERIALS AND METHODS

Materials. [^{3}H]IP_4 (17 Ci/mmol; 1 Ci = 37 GBq), [^{3}H]IP_6 (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 (M_r 3350) were obtained from Sigma. Concanavalin 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 phenylmethanesulfonyl 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 IP_4 and IP_6. Membrane binding of [^{3}H]IP_4 or [^{3}H]IP_6 was determined by incubating 50 μg of membranes with 0.030 μCi of [^{3}H]IP_4 or [^{3}H]IP_6 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. Nonspecific binding was determined by including 3 μM IP_4 or IP_6 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 [^{3}H]IP_4 or [^{3}H]IP_6 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

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Abbreviations: PKA, cyclic AMP-dependent protein kinase; IP_3, inositol 1,4,5-trisphosphate; IP_4, inositol 1,3,4,5-tetrakisphosphate; IP_6, inositol hexakisphosphate; IP_5, 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_r 3350) for 10 min at 0°C and centrifuged for 10–15 min at 12,000 × *g*. Supernatants were aspirated and pellets were solubilized as described for membranes. Nonspecific binding was determined by including 3 μM unlabeled IP_4 or IP_6 in the assays.

Synthesis of IP_4 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 $NaHCO_3$ (pH 8.5). After stirring at 4°C for 1.5 hr and overnight at 12°C, the IP_4 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 $MgCl_2$ and 1 mM $CaCl_2$, 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 IP_3 receptor adhered to the Con A column. The column was washed with 20 ml of homogenization buffer containing 1% CHAPS, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 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 IP_4 column adapted to the FPLC (dimensions, 10 cm × 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 IP_4 affinity column fractions with 10 mM $MgCl_2$, 50 μM ATP, and 5 μg of purified PKC or PKA per ml as described (8, 34, 35). For PKC phosphorylation, 1 mM $CaCl_2$, 5 μg of diacylglycerol per ml, and 50 μg of phosphatidylserine were included as described (36). Reactions were carried out at 30°C. No phosphorylation was detected in the absence of added kinase. After various times SDS sample buffer was added to stop phosphorylation and samples were separated by SDS/PAGE.

RESULTS

Purification of IP_4 and IP_6 Receptor Binding Proteins. Previously we observed [^{3}H]IP_4 binding to brain membranes and to solubilized and partially purified preparations (26, 27). Similar to the IP_3 receptor, the IP_4 receptor adheres to a heparin-agarose resin but, unlike the IP_3 receptor, IP_4 binding activity does not adsorb to Con A-Sepharose, permitting separation of the two receptors.

The binding specificity and affinity of [^{3}H]IP_4 to the solubilized preparation differ from the membrane preparation (26, 27); thus [^{3}H]IP_4 may bind to multiple sites in the membrane, or the IP_4 receptor may be allosterically coupled to other proteins in the membrane-bound state. Therefore, it is difficult to quantify the recovery of specific IP_4 receptors in the solubilization process. Of the total [^{3}H]IP_4 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 [^{3}H]IP_6 binding activity. The inositol phosphate specificity of solubilized [^{3}H]IP_6 binding sites resembles that of intact membranes (33). IP_6 displays a K_d of about 12 nM, with IP_5 and IP_4 being 2- to 3-fold less potent. IP_3 has only about 5% of the affinity of IP_6 for the binding sites. Similar to [^{3}H]IP_4 binding, ≈62% of [^{3}H]IP_6 binding is recovered in the solubilization process (Table 1).

[^{3}H]IP_4 and [^{3}H]IP_6 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 IP_4 and IP_6 binding activities with slightly more than 50% recovery of activity (Table 1). IP_4 and IP_6 binding activities flow through the Con A-Sepharose resin, which retains the IP_3 binding activity and provides an additional 3-fold increase in specific activity for IP_4 and IP_6 binding.

IP_4 and IP_6 binding activities have been separated using a P-1 tethered phosphodiester of IP_4 (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 IP_4 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 [^{3}H]IP_6 binding elutes first with ≈400 mM NaCl, followed by two successive, overlapping peaks enriched in [^{3}H]IP_4 binding, which elute between 600 and 900 mM NaCl (Fig. 2).

The inositol phosphate specificity of the first eluted peak is similar to [^{3}H]IP_6 binding in membranes and in crude solubilized preparations (Fig. 3). The K_d for IP_6 is 12 nM, with IP_5, IP_4, and IP_3 displaying approximately 50%, 30% and 5%, respectively, of the potency of IP_6. By contrast, the second and third peaks display a high affinity (K_d = 3–4 nM) and selectivity for IP_4. IP_4 is approximately 4, 10, and 1000 times more potent than IP_5, IP_6, and IP_3, respectively (Fig. 3).

The IP_4 affinity column provides a striking enrichment in IP_4 and IP_6 binding activities. The specific activity for IP_6 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 IP_4 binding are purified 100-fold compared with the Con A

Table 1. Purification of IP_4 and IP_6 receptors by affinity chromatography

Purification step	IP_4 binding	Yield, %	IP_6 binding	Yield, %
Membranes	1	100	4.3	100
Solubilized membranes	1.5	60	6.2	62
Heparin eluate	12	52	45	55
Con A flow-through	32	47	122	49
IP_4 column				
Peak 1	—	—	1950	41
Peaks 2 and 3	3640	35	—	—

Inositol phosphate binding activities were assayed by polyethylene glycol precipitation with 0.03 μCi of [^{3}H]IP_4 or [^{3}H]IP_6 at pH 7.4. Binding (B_{max}) is expressed as pmol/mg of protein. A single experiment, representative of four independent purifications, is depicted.

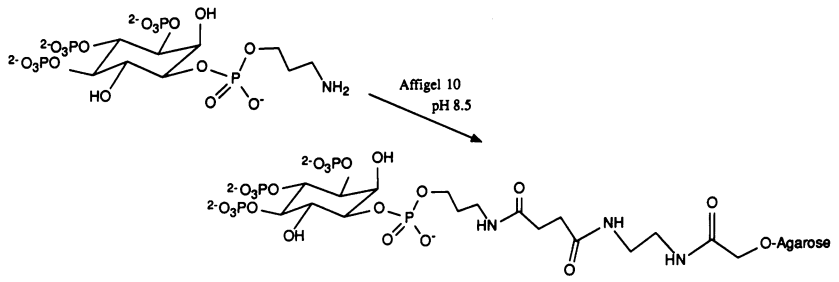

FIG. 1. Synthesis of IP_4 affinity resin by coupling of 1-*O*-(3-aminopropyl-1-phospho)-*myo*-inositol 3,4,5-trisphosphate to Affi-Gel 10.

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 IP_6 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 IP_6 receptor, we conducted size exclusion chromatography with an FPLC Superose 6 column. A single peak of $[^3H]IP_6$ 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

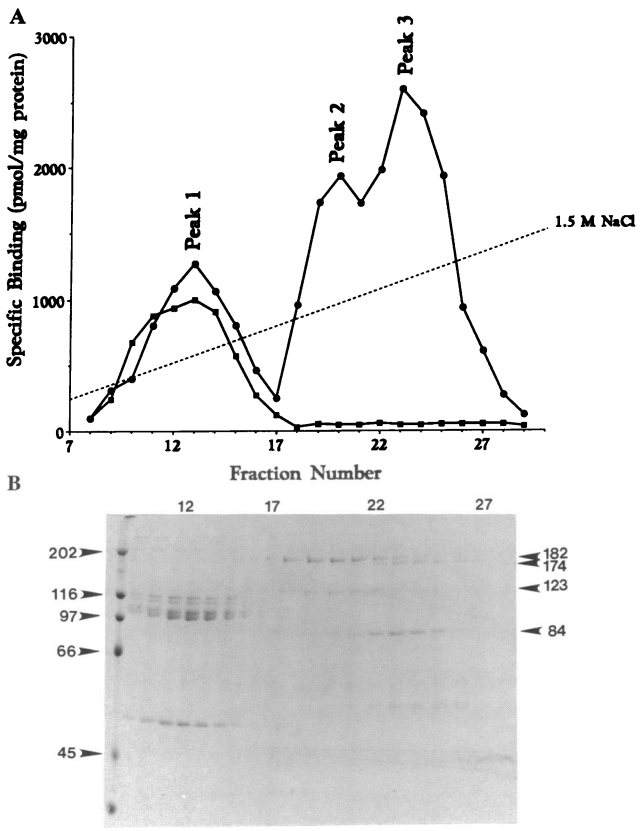

FIG. 2. (*A*) Elution profile of IP_6 and IP_4 receptors from the IP_4 affinity resin. Con A flow-through plus wash from 30 rat cerebella were chromatographed on an aminopropyl IP_4 Affi-Gel 10 column adapted to FPLC and eluted by a 0.15–1.5 M NaCl gradient in 1.5-ml fractions. Due to interference of detergent in protein assays, protein concentration was determined by comparison to bovine serum albumin staining on SDS/PAGE. Binding of $[^3H]IP_4$ and $[^3H]IP_6$ was assayed by polyethylene glycol precipitation with 0.03 μCi of $[^3H]IP_4$ (●) or $[^3H]IP_6$ (■) radiolabeled ligand and 50 μl of each column fraction. Nonspecific binding was determined with 3 μM unlabeled IP_4 or IP_6 and was <10% of total binding. A typical elution profile of five independent chromatographic runs is depicted. (*B*) SDS/PAGE analysis of IP_4 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.

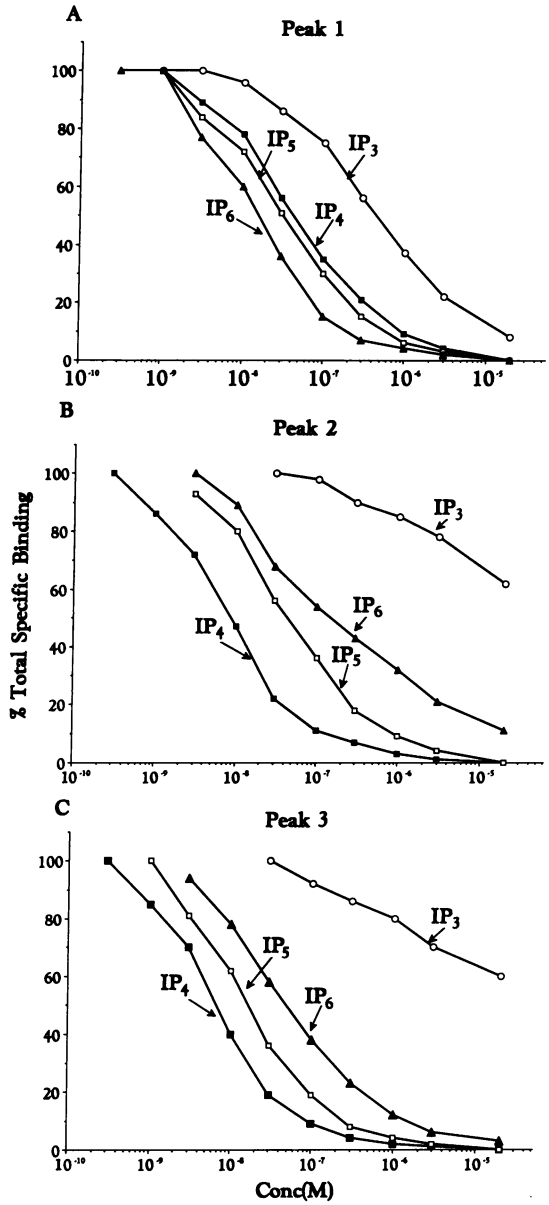

FIG. 3. Inositol phosphate specificity of IP_6 and IP_4 receptors. (*A*) Peak 1 from IP_4 affinity column eluting at 400 mM NaCl. (*B*) Peak 2 eluting at 750 mM NaCl. (*C*) Peak 3 eluting at 900 mM NaCl. Binding was assayed by polyethylene glycol precipitation using 0.03 μCi of $[^3H]IP_4$ and 30 μl of each fraction at pH 7.4. Data are means from three independent experiments performed in duplicate with standard errors of <10%. ○, IP_3; ■, IP_4; □, IP_5; ▲, IP_6.

cannot be resolved by cation- or anion-exchange chromatography. Together with the finding that the three bands emerge simultaneously from the IP_4 affinity chromatography column, we conclude that all three are components of the IP_6 receptor binding protein complex.

The IP_4 receptor peak that elutes first from the IP_4 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 IP_4 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 IP_4 and IP_6 Receptor Proteins. IP_4 and IP_6 receptor binding activities from the IP_4 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 IP_6 binding is 6.0–6.5. The two IP_4 binding fractions have pH optima of 5.0. Heparin potently inhibits IP_4 and IP_6 receptor binding (IC_{50} = 0.1 μg/ml). Ca^{2+} and Mg^{2+} enhance IP_4 and IP_6 binding to purified receptors 20–50% at 0.1 mM and inhibit binding 25–75% at 1 mM (data not shown).

To determine whether [^{3}H]IP_4 and [^{3}H]IP_6 binding might involve enzymes associated with inositol phosphate phosphorylation or dephosphorylation, we assayed each of the peaks from the IP_4 affinity column for phosphatase and kinase activity utilizing [^{3}H]IP_3 and [^{3}H]IP_4 as substrates. None of the three protein peaks displays any enrichment of these enzymatic activities (data not shown).

Like the IP_3 receptor (35), the IP_4 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 IP_4 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 IP_6 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 IP_4 and IP_6 receptor proteins. IP_5 has substantial affinity for the isolated binding proteins. Thus, these proteins might include physiological IP_5 receptors.

Successful purification employed an IP_4 affinity chromatography column that has substantial affinity for the IP_4 and IP_6 receptors. The utility of inositol phosphate affinity chromatography is also evident from our purification of IP_3 receptors with an IP_3 Affi-Gel affinity column (25).

The inositol phosphate specificity of the purified IP_6 receptor resembles the pattern for [^{3}H]IP_6 binding to brain membranes (33). This IP_6 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 IP_6 receptor protein physiologically interacts primarily with IP_6 or IP_5 is unclear, though it possesses about a 2-fold higher affinity for IP_6 than IP_5.

Nicoletti *et al.* (32) reported high affinity binding of [^{3}H]IP_6 in brain and pituitary membranes, which differs from the receptor we have purified. For instance, Nicoletti *et al.* (32) found IP_5 only 0.5% as potent as IP_6, whereas we observe only a 2-fold lower potency of IP_5. Moreover, the B_{max} and temperature dependence differ markedly for the two IP_6 binding sites.

We have isolated IP_4 receptor proteins of molecular sizes 182, 123, 174, and 84 kDa. These proteins display similar high

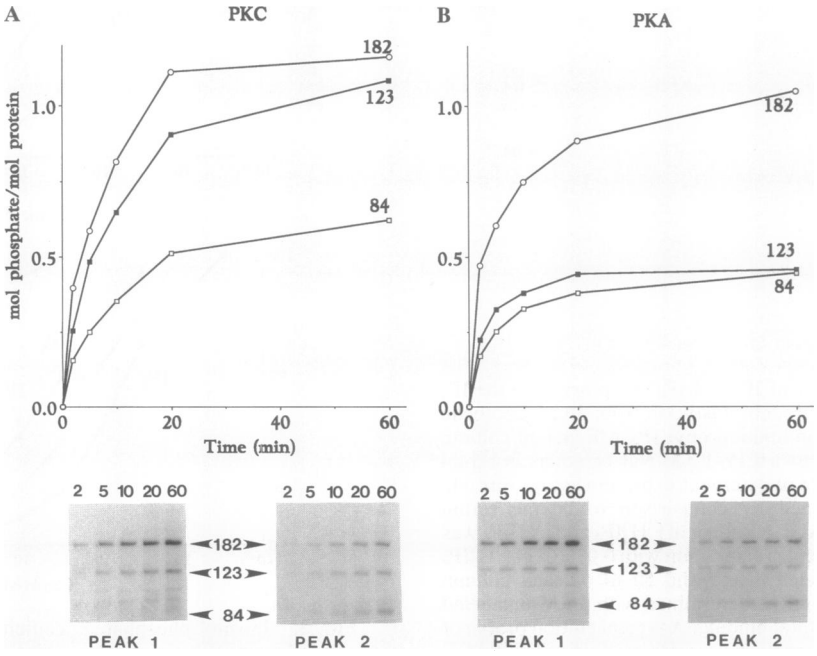

FIG. 4. Time course for phosphorylation of IP_4 receptors by PKA and PKC. Incorporation of ^{32}P from [^{32}P]ATP was assayed as described in the text. Aliquots (0.5 μg) of peak 2 or peak 3 from the IP_4 affinity column were incubated with the kinases for the time indicated and subjected to SDS/7.5% PAGE. Gels were dried, autoradiograms were prepared, and bands were excised and assayed to determine the extent of ^{32}P incorporation shown. Protein was quantified as in Fig. 1. Catalytic subunits of PKA and PKC were purified as described (8, 35). Sizes are indicated in kDa.

affinity for IP_4 (K_d = 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 posttranslational modifications of the IP_4 receptor proteins. Further characterization will determine the relationship of these protein subunits. Donie *et al.* (29) partially purified [^{3}H]IP_4 binding activity from porcine cerebellar membranes using heparin-agarose. Their binding site displays low nanomolar affinity for IP_4 with a somewhat different inositol phosphate specificity but a similar pH optimum. IP_4 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 IP_4 and IP_6 receptor proteins may clarify physiological roles of these inositol phosphates. Both have been reported to influence calcium disposition. IP_4 acts synergistically with IP_3 in regulating calcium release (17, 18) and IP_6 affects calcium accumulation in cultured brain cells (23). With IP_3 receptor protein reconstituted into lipid vesicles we demonstrated IP_3 stimulation of calcium flux (3). IP_3 inhibits [^{3}H]IP_4 binding potently in membranes. Following solubilization, though, this inhibition decreases dramatically, suggesting that IP_3 and IP_4 receptors interact allosterically, as has been recently proposed (36, 37). Similar reconstitution experiments for IP_4 and IP_6 receptors possibly together with IP_3 receptors may identify their roles in calcium regulation or in other cellular or extracellular actions.

We thank Dr. R. Huganir for advice in phosphorylation studies. G.D.P. thanks the Center for Biotechnology and the New York State Center for Science and Technology for financial support and Dr. J. F. Marecek (Chemical Synthesis Center, Stony Brook) for technical guidance. V.A.E. was a W. Burghardt Turner Fellow of the Graduate School of State University of New York, Stony Brook. This research was supported by U.S. Public Health Service Grants DA-00266 and MH-18501, Research Scientist Award DA-00074 to S.H.S., Predoctoral Fellowship MH-10018 to C.D.F., Training Grant GM-07309 to S.K.D., a grant from the International Life Sciences Institute, and a gift from Bristol-Myers-Squibb.

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