Structural and Functional Characterization of an Inositol Polyphosphate Receptor from Cerebellum*

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An inositol polyphosphate receptor has been purified from bovine cerebellum which consists of three different polypeptides with Mr of 111,000, 102,000, and 52,000. Negative staining electron microscopy reveals globular-like structures 10–13 nm in diameter. The receptor has a Stokes radius of 400,000 daltons as determined by molecular sieve high performance liquid chromatography. The receptor preparation binds inositol 1,3,4,5-tetrasphosphate, inositol hexaphosphate (or phytol), and inositol 1,4,5-trisphosphate (IP₃, IP₆, and IP₃, respectively) with submicromolar affinity (0.19, 0.15, and 0.54 μM, respectively) at conditions approximating physiological ionic strength and pH. The purified receptor preparation, when reconstituted into planar bilayers, displays ion channel activity, preferentially permeable to K⁺. Permeability ratios of the channel are P⁺/PNa⁺ ~5 and P⁺⁺/PCl⁻ ~19. In symmetrical 100 mM KCl, the channel is characterized by long open times (minutes) with a conductance of 7.2 picoSiemens. The channel is selectively modulated by IP₃. That is, at 1 μM IP₃, the mean open time decreased substantially to rapid flicker behavior and the channel is completely closed at 10 μM IP₃. IP₆ and IP₃ did not modulate the channel under similar conditions. Thus, the channel appears to be an IP₃-modulated K⁺ channel.

Hormone interaction with specific plasma membrane receptors trigger the G-protein mediated activation of a phosphatidylinositol specific phospholipase C which generates both inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol. Diacylglycerol activates protein kinase C, whereas IP₃ serves as a second messenger to mobilize calcium from intracellular stores. By this mechanism, the generation of IP₃ activates numerous Ca²⁺-dependent processes (1, 2).

Characterization of the molecular machinery involved in the IP₃-dependent Ca²⁺ release process has recently been achieved. An IP₃ receptor has been isolated from both cerebellum (3, 4) and smooth muscle (5). Comparison of the smooth muscle IP₃ receptor with the IP₃ receptor from cerebellum indicates that the two receptors are similar both structurally and functionally (6). Functional identity of the IP₃ receptor as an intracellular Ca²⁺ release channel has been suggested to act as an intracellular messenger (11–13). In this study, we describe the purification and characterization of a receptor from cerebellum which bound IP₃. After isolation, we found that it bound also IP₃ and IP₆. Reconstitution of this inositol polyphosphate receptor preparation into planar bilayers displays potassium channel activity which is selectively modulated by IP₃. A preliminary communication has appeared (14).

EXPERIMENTAL PROCEDURES

Materials

IP₃ was purchased from Calbiochem, IP₆ was from Boehringer Mannheim and IP₃ was from Sigma. [³H]IP₃ (17 Ci/mmol), [³H]IP₆ (17 Ci/mmol), and [³H]IP₃ (12 Ci/mmol) were from Du Pont-New England Nuclear. CHAPS and heparin-agarose were obtained from Sigma. High and low molecular weight standards for SDS-PAGE were from BioRad. Gel filtration molecular weight standards and phenyl-Sepharose 4B were from Pharmacia LKB Biotechnology Inc. Bovine brain cerebellum was quick-frozen with liquid nitrogen and stored at −80 °C.

Protein Assay

Protein determination was performed by the method of Kaplan (16) or by scanning densitometry of protein bands on Coomassie Blue-R-250 stained SDS-polyacrylamide gels using an automated gel analysis plus image processing system (Technology Resources, Nashville, TN). Bovine serum albumin was used as standard.

Electron Microscopy

Negative staining of the purified receptor was performed as described previously (5).
Unles indicated otherwise, all solutions contained protease inhibitors obtained from Sigma (pepinstatin A at 0.5 μg/ml, leupeptin at 0.5 μg/ml, and aprotonin at 0.21 μg/ml), and the pH of solutions was adjusted at room temperature. All purification steps and binding assays were performed at 4°C.

Isolation of Bovine Cerebellum Microsomes

Twelve 5-g portions of frozen bovine cerebellum were each thawed for 10 min at 30 °C in ice-cold buffer A (50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM DTT) contained in Beckman JA-10 centrifuge bottles and homogenized for 15 s at setting 6 using a polytron homogenizer (PT 20). The homogenate were then centrifuged for 1 h at 14,000 rpm using a Beckman JA-14 rotor. The clear supernatants were adjusted and the pellets resuspended in 250 ml buffer A containing 150 mM NaCl. The suspensions were homogenized for 10 s at setting 6 using the polytron and centrifuged as described above. The pellets were resuspended to a final volume of 250 ml in buffer A and stored at −80°C. Approximately 2400 mg of microsomal protein was obtained from 60 g wet weight of cerebellum.

\[ \gamma^3H]IP_3, \gamma^3H]IP_4, \text{ and } \gamma^3H]IP_6 \text{ Binding Assays} \]

For Scatchard analyses, \( \gamma^3H]IP_3, \gamma^3H]IP_4, \text{ and } \gamma^3H]IP_6 \) binding (specific activity = 17, 17, and 12 Ci/mmol, respectively) were used to monitor purification of the receptor. Binding of \( \gamma^3H]IP_3, \gamma^3H]IP_4, \text{ and } \gamma^3H]IP_6 \) was performed as described previously (5) except that, in order to conserve ligands, nonspecific binding was determined in the presence of heparin (100 μg/ml) which gave similar values to that determined in the presence of excess IP_3, IP_4, and IP_6.

For competition studies of \( \gamma^3H]IP_3, \gamma^3H]IP_4, \text{ and } \gamma^3H]IP_6 \) binding to purified receptor, 0.2 or 1.0 μg of receptor protein was assayed in 100 μl of binding medium composed of 25 mM Na-Hepes pH 7.1 (pH 7.4 on ice), 1 mM EDTA, 1 mM DTT, 1% CHAPS, 5 mg/ml equine IgG (Sigma), 10 mM \( \text{NaCl} \) (17 Ci/mmol) in the presence of 0–5 μM unlabeled ligand and ±100 mM KCl. The receptor was coprecipitated with IgG using 6% (w/v) polyethylene glycol and pelleted by centrifugation as described previously (6). Nonspecific counts were determined by omission of the purified receptor from the binding medium. Competition curves were generated by fitting the data to a generalized ligand binding equation:

\[ y = ax/(b + x^n) \]

where a is the maximal binding (normalized to 100%), b is the IC_{50}, and c is the slope factor where a value of 1 indicates normal hyperbolic binding while values greater or less than 1 suggest either positive or negative cooperativity, respectively (see Ref. 29).

For Scatchard analyses, \( \gamma^3H]IP_3, \gamma^3H]IP_4, \text{ and } \gamma^3H]IP_6 \) stock solutions were either used as obtained from Du Pont-New England Nuclear (0.59, 0.59, and 0.80 μM, respectively) or were supplemented with 1% CHAPS, 5% BSA, and 100 mM NaCl to give stock concentrations of 6.9 μM for IP_3, and 4.0 μM for IP_4. The binding studies were then carried out as a function of ligand concentration by addition of increasing amounts of radioligand solution. The concentrations of the respective ligands used in all binding assays were calculated from information provided by the suppliers of radioactive and nonradioactive inositol phosphates.

Purification of the Inositol Polyphosphate Receptor

Solubilization of the Microsomes—Frozen microsomes from 120 g (wet weight) of cerebellum were thawed and mixed with 1500 ml of buffer B (50 mM Tris-Cl (pH 7.4), 1 mM EDTA, and 1 mM DTT). Solubilization was initiated by addition of 1000 μl buffer B containing 60 g of CHAPS (final concentration = 2%, w/v). After gentle stirring for 1 h, the suspension was centrifuged for 1.5 h at 14,000 rpm using a Beckman JA-14 rotor. The pellets were discarded, and the supernatant was used for purification of the inositol polyphosphate receptor.

Purification of the Receptor—The supernatant was adjusted to 200 mM with NaCl and gently mixed with 85 ml of heparin-agarose (which had been equilibrated with H_2O). After 2.5 h, the gel was collected by vacuum filtration, washed with 400 ml of buffer B containing 1% (w/v) CHAPS and 200 mM NaCl, and poured into a 2.5-cm inner diameter column. The receptor was eluted with the same buffer containing 800 mM NaCl and 8 ml of fractions were collected. The five peak fractions (Nos. 12–16) containing IP_3 binding activity were pooled. The pooled fractions from the heparin-agarose column were dialyzed overnight against 1100 ml of buffer C (20 mM Tris-Cl (pH 7.4), 1 mM DTT, and 1 mM EDTA containing 1.0% (w/v) CHAPS). The dialyate was diluted with an equal volume of buffer C and centrifuged for 30 min at 20,000 rpm using a Beckman Ti 70 rotor to remove aggregated protein. The supernatant was loaded onto a 15 ml column of DEAE-Sepharose-6B (1.5 cm inner diameter) at a flow rate of 0.5 ml/min, washed with buffer C, and eluted with a 150-ml gradient of 0–250 mM NaCl in buffer C, 7.5-ml fractions were collected. Peak fractions containing IP_3 binding activity (fractions 11–14) were pooled, frozen in liquid nitrogen, and stored at −80°C.

After thawing, the pooled fractions were loaded at 0.5 ml/min onto a second heparin agarose column (3 ml, 1.0 cm inner diameter) which had been equilibrated in buffer D (50 mM Tris-Cl (pH 8.3), 1 mM EDTA, 1 mM DTT) containing 1.0% CHAPS. Elution of the receptor was achieved with a 40-ml gradient of 0.2–0.8 M NaCl in the same buffer, and fractions of 2 ml were collected. Fractions 8–15 containing the receptor were pooled and adjusted to 2.5 M NaCl. The sample was then applied onto a 2.0-ml column of phenyl-Sepharose 4B which had been equilibrated with buffer D containing 2 M NaCl and 1.0% CHAPS. The column was washed with 6 ml of the same buffer containing 2 M NaCl and the receptor obtained by sequential elution with 4 ml each of 1, 2, 3, and 4% (w/v) CHAPS in buffer D (see Fig. 3B). The 3B fractions obtained (2 ml containing CHAPS concentrations of 2% or more, were pooled. The pooled fractions from the phenyl-Sepharose column were concentrated (Centricon-30) to 1–2 ml and loaded onto a TSK gel G3000SW column (60 cm × 21 mm) and eluted at 1.5 ml/min with buffer E (50 mM imidazole (pH 6.9), 100 mM NaCl, 1% CHAPS, 1 mM EDTA, 1 mM DTT). Fractions of 2.4 ml were collected and elution was monitored by A_{280}. Peak fractions (43–46) which contained the IP_3 binding activity, were pooled, concentrated (Centricon C-50), and stored frozen at −80°C. In practice, a single preparation was performed over a period of five days.

Reconstitution into Planar Bilayers and Channel Measurements

Preparation of Lipid/protein and Lipid Vesicles—The lipid used throughout this study was a lipid mixture: soybean lipid (Sigma, type II-S, acetone-washed) with cholesterol (Fluka) in a weight ratio of 24:1. Lipid vesicles and lipid/protein vesicles were prepared in either 100 mM KCl or 100 mM NaCl buffer to pH 7.4 by 10 mM Hepes-Tris buffer. Treatments to other salt concentrations, i.e. 600 mM KCl or additional CaCl_2, were done after bilayer formation. Lipid vesicles were prepared by resuspending a thin film of 10 mg of lipid dried under nitrogen in 10 ml of buffer (15). Protein/lipid vesicles preparation: 10 μl of receptor protein (0.54 mg/ml), 6 mg/ml CHAPS, and 3 mg/ml soybean lipid were suspended in 1 ml buffer for thin film formation. Planar bilayer lipid membranes (Bio-Beads (Bio-Rad) were added (0.23 g/ml), and the flask rotated for 1 h, followed by a second 1-h rotation with fresh 0.33 g/ml Bio-Beads. The sample was stored on ice until use (up to 3 h).

Planar Lipid/protein Bilayers Formation—Planar bilayers were formed from vesicles of defined lipid protein ratio, according to the septum-supported vesicle-derived bilayer technique (15). Briefly, planar bilayers (aperture size of 0.18 mm) were formed by apposition of two vesicle derived monolayers. Both cis (protein-containing side) and trans chambers contain 1 ml of solution. Five (Figs. 10 and 12) and 2.5 μl (Fig. 11) of fresh protein lipid vesicle preparation was diluted into 2 ml of fresh lipid vesicle preparation to provide a working suspension of proteolipid vesicles. Using the molecular weights, 400,000 for IP_3 receptor and 750 for soybean phospholipid, the two above protein concentrations correspond to final molar ratios of protein/lipid in vesicles of 2.2 and 1.1-10^{-4}, respectively, as given in the Fig. legends. IP_3 (Boehringer Mannheim) was applied in 1-μl aliquots directly to the membrane on the cis side via a steel tube. The tube, of inner diameter 0.85 mm, was adjusted with its end to the membrane (center to center) at a distance of 0.15 mm. It could be removed for refilling and accurately replaced in the same position. Using fructose (concentration of 0.66 ng/ml, 1 μl applied) for calibration, the time for half-maximal response of induced current was 6 ± 1 s. The response showed a plateau between 30 s and 3 min followed by a slow decay (60% after 12 ± 1 min) due to diffusion loss of the applied
valinomycin into the bulk solution (10).

Electrical Measurements and Data Processing—Electrical contact with the solution was made via Ag/AgCl electrodes. Voltage is expressed as the voltage applied to the cis solution. The voltage signal across the feedback resistance (10 kΩ) of the current-measuring operational amplifier was filtered at 3 kHz and stored on a pulse code-modulated audio tape recorder modified to accept dc signals.

RESULTS

Purification and Characterization of the Inositol Polyphosphate Receptor—Competition of [3H]Ins(1,3,4,5)P₄ binding to bovine brain cerebellum microsomes at pH 8.9 detects low affinity Ins(1,3,4,5)P₄ binding with a Ka in the range of ~1 μM (Fig. 1).

To further characterize this low affinity IP₄ receptor, its purification was achieved as described under "Experimental Procedures" and summarized in Table I. We have designated this receptor the inositol polyphosphate receptor since it also binds IP₆ and IP₃ (see below). Microsomes from cerebellum were solubilized with CHAPS, and the receptor was purified using a combination of column chromatography procedures. Approximately 80% of IP₄ binding activity was solubilized by extraction of cerebellum microsomes with 2% CHAPS. The [3H]IP₄ binding activity was then concentrated and enriched by absorption on heparin-agarose and elution with buffer containing high salt (Table I). After dialysis to lower the NaCl concentration, the IP₄ binding activity was purified by sequential chromatography on DEAE-Sepharose 6B, a second heparin-agarose column, and phenyl-Sepharose 4B. The final step made use of a TSK gel G3000SW gel filtration column. The receptor is enriched approximately 140-fold from the solubilized microsomes, with an estimated recovery of 1%. A significant loss of IP₄ binding equivalents is sustained prior to the DEAE-Sepharose 6B step (Table I). This is attributable in part to co-precipitation of IP₄ binding activity (~50%) together with contaminating proteins during the dialysis step preceding the DEAE-Sepharose 6B chromatography.

The protein profile of the fractions obtained in the purification procedure was characterized by SDS-PAGE (Fig. 2). The purified receptor consists of three polypeptides with apparent molecular weights of 111,000 ± 10 (mean ± S.D., n = 3), 102 ± 1.1 (mean ± S.D., n = 3), and 52.0 ± 1.0 (mean ± S.D., n = 3). In the enriched fractions of the receptor from the phenyl-Sepharose (Fig. 3B, fractions 7–9) and TSK Gel gel filtration columns (Fig. 4B, fractions 43–45), a constant ratio of the three polypeptide bands is observed, as determined by densitometry, which correlates with [3H]Ins(1,3,4,5)P₄ binding. These findings suggest that the receptor is a heterooligomeric complex of the three bands. Pretreatment of the purified receptor with 2-mercaptoethanol and heating (100 °C, 1 min) did not change the electrophoretic profile (not shown).

The three bands characteristic of the receptor are co-enriched at each step in the purification (Fig. 2, lanes 2–6). The enrichment, observed in each of 10 preparations, correlates with the determinations of IP₄ binding activity (Table I), with the exception that there is a decrease in specific binding in the DEAE-Sepharose 6B pool compared with the pooled

![Fig. 1.](image)

**FIG. 1.** [3H]IP₄ binding to bovine cerebellum microsomes and competition by unlabeled IP₆. [3H]IP₄ binding was determined with 0.1 mg of cerebellum microsomal protein in 0.1 ml of binding medium composed of 50 mM Tris-Cl (pH 8.9), 1 mM EDTA, 1 mM DTT, 10 nM [3H]IP₄ (17 Ci/mmol), and the indicated amounts of unlabeled IP₆. Nonspecific binding, determined in the presence of 100 μg/ml heparin, accounted for 2% binding, whereas total binding was 5% of the total radiolabel. An IC₅₀ value of 0.83 μM for unlabeled IP₆ indicates the presence of low affinity IP₆ binding in these microsomes. The curve was generated by fitting the data to the equation y = a/(1 + (x/b)) by nonlinear regression (Marquardt-Levenberg algorithm) (see "Experimental Procedures").

| Fraction | Protein mg | IP₄ binding * Pmol | Purification yield % |
|----------|-----------|-------------------|---------------------|
| Solubilized microsomes | 4827 | 1623 | 0.34 | 100 |
| Supernatant | 1177 | 1302 | 1.11 | 80 |
| First heparin column pool | 152 | 902 | 5.9 | 17.4 |
| DEAE-Sepharose pool | 19.4 | 76 | 3.9 | 11.5 |
| Second heparin column pool | 8.0 | 47 | 5.9 | 17.4 |
| Phenyl-Sepharose pool | 2.2 | 37.4 | 17.0 | 50.0 |
| TSK gel G3000SW pool | 0.36 | 16.7 | 46.4 | 136.5 |

* The concentration of [3H]IP₄ used to determine binding was 10 nM. Binding of [3H]IP₄ was found to be inhibited by increasing concentrations of NaCl (C. C. Chadwick and S. Fleischer, unpublished results). Therefore, the concentration of NaCl in each assay was adjusted to 40 mM.

**Fig. 2.** Protein profile of fractions obtained in the purification of the inositol polyphosphate receptor. SDS-PAGE was carried out on a 7.5% polyacrylamide gel using the buffer system of Laemmli (24). Samples were treated with 1% 2-mercaptoethanol, 2% SDS at room temperature (5 min) prior to electrophoresis. The gel was stained with Coomassie Blue R-250. Lane 1, supernatant extract of cerebellum microsomes with 2% CHAPS; lane 2, pooled fractions from the first heparin-agarose column; lane 3, pooled fractions obtained from the DEAE-Sepharose 6B column; lane 4, pooled fractions obtained from the second heparin-agarose column; lane 5, pooled fractions obtained from the phenyl-Sepharose 4B column; lane 6, pooled and concentrated fractions from the TSK gel G3000SW column. The protein loaded onto each lane was 1.25 μg as determined by the Kaplan and Pedersen method (16). The numbers on the left of the gel indicate the positions of M₅, standards: myosin, M₅ = 200,000; β-galactosidase, M₅ = 116,250; phosphorylase b, M₅ = 97,400; bovine serum albumin, M₅ = 66,200; ovalbumin, M₅ = 45,000. The three bands referable to the inositol polyphosphate receptor are indicated by arrows.
The molar ratio of 111-, 102-, and 52-kDa subunits, estimated by densitometric scanning of Coomassie Blue-stained SDS-PAGE gels of four purified receptor preparations is 1:0.3:4 ± 0.8:2.4 ± 0.5 (n = 4 for each band). Molecular sieve HPLC reveals a molecular weight of ~400,000 for the purified receptor in 1% CHAPS (Fig. 5). Negative staining electron microscopy reveals uniform globular-like structures of approximately 10-13 nm in diameter (Fig. 6).

Characterization of IP₃, IP₄, and IP₆ Binding to the Purified Receptor—Binding of [³H]Ins(1,3,4,5)P₄ to the purified receptor is not unique, since competition is observed not only with IP₃, but also with IP₄ (Fig. 7A) and IP₆ (Fig. 7B). The binding by IP₄ and IP₆ at pH 7.4 appears to be high affinity but is attenuated by ionic strength. In a buffer of 25 mM Na-Hepes (pH 7.4), Ins(1,3,4,5)IP₄, and InsP₆ similarly compete for binding with IC₅₀ values of ~25-35 nM. However, binding is weaker when measured under conditions approximating more physiological ionic strength (100 mM KCl, 25 mM sodium-Hepes (pH 7.4)). The estimated IC₅₀ values for both Ins(1,3,4,5)P₄ and InsP₆ are substantially increased (>5-fold) to 150 nM (Fig. 7A and Table II). Ins(1,4,5)P₃ also inhibits [³H]Ins(1,3,4,5)P₄ binding (Fig. 7B). The estimated IC₅₀ value for Ins(1,4,5)P₃ increases about 2.5-fold in going from the lower ionic strength to physiological ionic strength at pH 7.4. The relatively higher IC₅₀ values under conditions approximating physiological pH and ionic strength, indicate that in situ the receptor has weaker affinity for these inositol polyphosphate ligands.

Binding parameters of the purified receptor were also obtained from Scatchard analyses (Fig. 8) for the three inositol polyphosphates, at conditions used to monitor purification of the receptor, i.e. pH 8.9 and 55 mM salt. Binding is characterized by a Kᵦ of 0.64 µM for Ins(1,3,4,5)P₄, Kᵦ = 0.49 µM for Ins(1,4,5)P₃, and Kᵦ = 0.12 µM for InsP₆ (Table III). The Kᵦ for Ins(1,3,4,5)P₄ binding to the purified receptor (0.64 µM) is in the same range as that estimated for Ins(1,3,4,5)P₄ binding to the starting microsomes (~0.8 µM, Fig. 1) when measured at comparable conditions. Thus, the binding affinity with regard to Ins(1,3,4,5)P₄ binding is essentially unchanged by purification.

Binding of [³H]Ins(1,3,4,5)P₄ and [³H]Ins(1,4,5)P₃ at pH 8.9 and 55 mM salt are inhibited by ATP (Kᵦ ~100 µM), GTP (Kᵦ ~100 µM), and hepamin (Kᵦ ~1 µg/ml); the Kᵦ values are about the same for both ligands. Ins(1)P and Ins(1,4)P₂ do not effectively compete for either Ins(1,4,5)P₃ or Ins(1,3,4,5)P₄ binding (Kᵦ >100 µM for both).

The finding that the receptor also binds IP₃ and IP₆ was unexpected. The previously isolated IP₃ receptor from cerebellum is characterized by a polypeptide with a molecular weight of 260,000 as determined by SDS-PAGE which binds IP₃ with a Kᵦ in the nanomolar concentration range (Refs. 3-6). Scatchard analysis of IP₃ binding to cerebellum microsomes is shown in
Purification of an Inositol Polyphosphate Receptor

The purified receptor preparation was recon-
stituted into liposome vesicles from which planar membranes were formed (see "Experimental Procedures"). Ion channel activi-
ties were clearly observed in 45 out of 60 membranes and were dependent on the presence of the receptor, in contrast to lipid membrane controls which showed no channel activity at all. Fig. 10 shows bursts of channel events of about 10-s duration which are typical for a KCl/NaCl gradient (100/100 mM) with positive potential applied to the cis side containing KCl (50 mV in Fig. 10). The upper burst was observed just

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**Table II**

| Ligand | Low salt | Isotonic salt |
|--------|----------|---------------|
|        | $IC_{50}$ | Slope factor | $IC_{50}$ | Slope factor |
| $IP_3$ | (0.20–0.26) | 0.64 | 0.58 | 0.65 |
| $IP_4$ | (0.025–0.038) | 0.82 | 0.19 | 0.81 |
| $IP_6$ | (0.014–0.033) | 0.56 | 0.58 | 0.94 |

**Fig. 5.** Molecular weight determination of inositol polyphosphate receptor by gel filtration HPLC. Molecular weight estimate of the purified inositol polyphosphate receptor by gel filtration HPLC. The purified receptor (25 μg of protein) was applied in a volume of 0.10 ml to a TSK gel G4000SWXL column (0.75 x 30 cm) pre-equilibrated in 20 mM imidazole CI (pH 6.8), 250 mM NaCl, 2 mM DTT, and 1% CHAPS. The flow rate was 1.0 ml/min and absorbance was monitored at 280 nm. M, standards which were chromatographed in the same buffer without CHAPS included: thyroglobulin, 669,000; ferritin, 440,000; and catalase, 232,000. The elution position of standards is indicated by the closed circles. The elution profile of the receptor is shown in the figure. An $M_1$ of 400,000 was estimated for the receptor.

**Fig. 6.** Electron microscopy of inositol polyphosphate receptor. Electron micrograph of the purified receptor obtained after negative staining with uranyl acetate. The receptor has a diameter of 10–13 nm.

**Fig. 7.** Competition of $[^{3}H]IP_3$ binding by $IP_3$, $IP_4$, and $IP_6$ at neutral pH (7.4). A: $IP_3$ (○) and $IP_6$ (●) at low salt (25.0 mM NaHEPES); $IP_3$ (□) and $IP_6$ (▲) at "isotonic salt" concentration (25 mM NaHEPES + 100 mM KCl). B: $IP_4$ at low salt (□) and isotonic salt (▲). $[^{3}H]IP_3$ (10 nM) was supplemented with cold ligand as indicated in the figure. Data from two such studies are summarized in Table II. The percentage of bound $[^{3}H]IP_3$ relative to total radiolabel ranged from 10 (at low ionic strength) to 3% (in isotonic salt). Nonspecific binding varied from 3% (low ionic strength) to less than 1% (isotonic salt).
Binding was equivalent. The percentage of bound ligand relative to the total radioligand ranged from 0.97 to 0.99 for IP₃ binding, 0.98 to 0.99 for IP₂ binding, and 0.99 to 0.95 for IP₁ binding. The correlation coefficients ranged from 0.99 to 0.94 for analyses of IP₃ binding, 0.97 to 0.99 for IP₂ binding, and 0.99 to 0.95 for IP₁ binding. The percentage of bound ligand relative to the total radioligand ranged from 3.8 to 8% for [³H]IP₃, 2 to 6.8% for [³H]IP₂, and 4.5 to 15% for IP₁. Nonspecific binding for all ligands was 2% of the total. Binding parameters from three or more experiments with three different preparations and assay conditions are summarized in Table III.

TABLE III
Scatchard analysis of binding data to the purified receptor

| Ligand | Kd (µM) | Bmax (nmol/mg) | n | Correlation coefficient |
|--------|---------|----------------|----|------------------------|
| IP₁    | 0.64 ± 0.21 | 3.0 ± 0.33     | 6  | 0.94-0.99               |
| IP₂    | 0.12 ± 0.03 | 1.29 ± 0.67    | 4  | 0.96-0.99               |

after bilayer formation and the middle burst 30 min later with 12 bursts of similar duration in the intermediate time. These types of bursts were observed in three out of three membranes studied at conditions of Fig. 10. Occasionally, a second class of bursts appeared with a much higher transition frequency as shown in the lower trace of Fig. 10. The mean open time of the channel was strongly dependent on ionic conditions. At symmetrical KCl (100 mM), channel open times were much longer, in the 1–10-min time range (cf. lowest trace in Fig. 11). Under these conditions, the channel showed a constant conductance of 7.2 ± 0.8 pS in the range of −100 to +100 mV applied potential and no significant dependence of mean open time with applied voltage (data not shown).

IP₁ was found to close the channel. The long mean open time of the channel in symmetrical 100 mM KCl was used to study effects of inositol polyphosphates on this channel activity. A key finding is illustrated in Fig. 11. It shows a continuous record where channel activity was completely blocked by 10 µM IP₁ (Ins(1,3,4,5)IP₃) when applied close to the membrane (cis side with 1 µl of 10 µM IP₁, using a precisely readable tube, see "Experimental Procedures"). Upon tube removal and 30-s stirring (dilution of IP₁ to ~10 nM), channel activity reappeared. Channel blockage by 10 µM IP₁ and reopening upon IP₁ dilution was repeated three times; two additional cycles are shown in the figure. The final trace (note the 3-fold compressed time scale) shows that, without application of 10 µM IP₁, the channel remained open for more than 15 min with occasional sojourns to the closed state.

Complete blockage by 10 µM IP₁ was observed in four out of four planar bilayer membranes (Fig. 11). Application of 10 µM IP₁ (Ins(1,3,4,5)IP₃) or 10 µM IP₂ at conditions of Fig. 11 did not activate additional channels or block the observed channels. In the presence of 1 µM IP₁ (conditions of Fig. 11) the long lasting open channel events were not completely blocked, but convert to flicker behavior, i.e. repetitive transitions between open and closed states, albeit similarly long lasting trains of rapid closing and reopening events (1–10 min). Such activity is exemplified in Fig. 12a, where it has been employed to determine the permeability ratio Pₖ⁺/Pₐ⁻ of the channel. The data were obtained in the presence of 1 µM IP₁ and a KCl gradient (500/100 mM). Open channel currents of the traces in Fig. 12a are plotted against applied voltage (Fig. 12b), yielding an estimate for the reversal potential of −35 ± 2 mV. Using the common constant field approximation (17), a permeability ratio, Pₖ⁺/Pₐ⁻ of ~19 is obtained, indicating a high selectivity of K⁺ ions against Cl⁻ ions. A second membrane at the same conditions yielded virtually the same...
We have purified an inositol polyphosphate receptor from bovine cerebellum which binds IP₄, IP₃, and IP₅. The receptor preparation, when reconstituted into planar bilayers, displays channel activity. IP₄ induces rapid channel flicker at 1 µM concentration and closes the channel at 10 µM. IP₃ or IP₅ are without effect under similar conditions.

The receptor has been enriched about 140-fold in IP₃ binding from the starting cerebellum microsomes (Table I) where it appears to exist at higher concentration by an order of magnitude than the IP₃ receptor described previously (3-5). This would be compatible with the minimal molecular weight of about 500,000, assuming that each of the peptides had the same extinction coefficient with Coomassie Blue and that the electrophoretic mobility obtained by SDS-PAGE provides reliable estimates of molecular weight. Our data suggest specific association of the 111,000, 102,000, and 52,000 bands as observed by SDS-PAGE (Fig. 2). The complex contains more than the standard error of the means. Connection of data points by the dashed line (hand drawn) yield a reversal potential of -35 ± 2 mV.

The Stokes radius of the purified receptor solubilized in CHAPS was determined by HPLC gel filtration to be about 100 nm. The channel currents observed in a KCl/NaCl gradient (100/100 mM) as shown in Fig. 10 for +50 mV applied to the KCl side, a reversal potential of -35 to -40 mV was obtained. From this value and from the permeability ratio P₄/P₃ of -1.1 and IC₅₀ (25 mM), 5-10-fold higher affinity is observed for IP₃ and IPS, respectively. At lower ionic strength, the purified receptor has binding affinity (IC₅₀ [3H]IP₄) in the range of -0.15, 0.2, and 0.5 µM for IP₃, IP₄, IP₅, respectively. At lower ionic strength (25 mM), 5-10-fold higher affinity is observed for IP₄ and IP₅ and >2-fold for IP₃ binding (Fig. 11 and Table II). Heparin markedly blocks the binding of IP₃, IP₄, and IP₅; inositol 1-phosphate and inositol 1,4-diphosphate do not effectively compete for binding of either IP₄ or IP₅.

The purified receptor preparation consists of three bands with apparent molecular weights of 111,000, 102,000, and 52,000 as determined by SDS-PAGE (Fig. 2). The complex has an estimated stoichiometry of 1:3:2. This would be compatible with the minimal molecular weight of about 500,000, assuming that each of the peptides had the same extinction coefficient with Coomassie Blue and that the electrophoretic mobility obtained by SDS-PAGE provides reliable estimates of molecular weight. Our data suggest specific association of the three polypeptides as a receptor complex: 1) the coenrichment of the 111,000, 102,000, and 52,000 bands as determined by SDS-PAGE (Fig. 2); and 3) the uniform size of the particles observed by electron microscopy (Fig. 6).
\[ \text{purification of an inositol polyphosphate receptor} \]

-400,000 (Fig. 5). Negative staining electron microscopy reveals a globular structure with a diameter of 10–13 nm (Fig. 6), consistent with a molecular weight in the range of 400,000. A receptor with \( M_r \) of 400,000, which binds one mole equivalent of ligand, has an expected \( B_{\text{max}} \) of 2.5 nmol/mg. This is in the range of \( B_{\text{max}} \) values determined for IP₃, but is somewhat lower for IP₄ and IP₅ (Table III).

The reconstitution experiments and channel measurements provide evidence that the purified receptor preparation is associated with ion channel activity. Channel currents were strictly dependent on the presence of the receptor preparation. We find that IP₃ induces channel flickering in the concentration range of the binding \( K_d \) (1–1 μM), with complete blockage at 10 μM IP₃ concentration. For initial classification of this channel, we studied permeability ratios of physiological ions (K⁺, Cl⁻, Na⁺, Ca²⁺), which indicate that the channel may be classified as a potassium channel, although residual Ca²⁺ permeability cannot be excluded, at present. The localization of the receptor in the cell remains to be determined. The K⁺ permeability and sensitivity of its open time to asymmetric Na⁺ and K⁺ across the membrane might indicate that the channel is of plasma membrane origin. In this first study, the channel characterization was limited to show that the receptor preparation forms an ion channel after reconstitution into planar bilayers. Although inactivation of the channel by IP₃ is reported here, the nature of activation of the channel, i.e., what turns it on and why it is selectively modulated by IP₃, remains to be discerned.

IP₃ has been implicated in intracellular Ca²⁺ mobilization in a diversity of cell types and has been shown to release Ca²⁺ from microsomes derived from cerebellum (18–20), smooth muscle (21), and platelets (22). A high affinity IP₃ receptor \( (K_d = 2–80 \text{ nM}) \) has been isolated previously from both cerebellum (3, 4) and smooth muscle (5). The cerebellum IP₃ receptor has been cloned and shown to have a protomer molecular weight of 313,000 (4, 6, 25). The smooth muscle IP₃ receptor is structurally and functionally similar to the cerebellum receptor (6). The cerebellum IP₃ receptor has been implicated as a Ca²⁺ channel, since IP₃ enhances Ca²⁺ efflux from vesicles containing the reconstituted receptor (7, 8). More recently, we have reconstituted the purified IP₃ receptor from smooth muscle into planar lipid bilayers and have directly demonstrated it to be an IP₃-activated Ca²⁺ channel (10). Thus, the high affinity IP₃ receptor from cerebellum (3, 4) and smooth muscle (5) is an IP₃-activated channel responsible for mediating release of intracellular Ca²⁺.

There is evidence that Ins(1,3,4,5)P₄ may also play a role in the mobilization of intracellular Ca²⁺. IP₄ appears to augment IP₃-dependent Ca²⁺ mobilization in mouse lachrymal acinar cells (12, 13). Ca²⁺ release from cerebellum microsomes has been reported to be triggered both by IP₃ (23) and IP₄ (19). IP₄ has been reported to regulate K⁺ channels (19). IP₄ has been reported to regulate K⁺ channels and IP₄ receptors isolated previously from cerebellum (3, 4) and smooth muscle (5). The high affinity IP₃ receptor is a tetramer of a single polypeptide chain which has a protomer molecular weight of 313,000 (4, 6, 25). Morphologically, the IP₃ receptor exhibits 4-fold symmetry, appearing as a pinwheel with four arms radiating from a central hub (5). The inositol polyphosphate receptor is a heterooligomer consisting of three polypeptides. It is spherical and much smaller in size (10–13 nm, Fig. 5) than the IP₃ receptor which has dimensions of 25 × 25 × ~10 nm (5). We estimate that in cerebellum, the inositol polyphosphate receptor is capable of binding about 20-fold more IP₃ than the high affinity IP₃ receptor (Fig. 9).

Significantly, IP₃ kinase and IP₃ phosphatase activities (either Ca²⁺/calmodulin-dependent or -independent) are not detected in the purified inositol polyphosphate receptor, although readily detected in the first heparin pooled fraction (5). Values for intracellular IP₃ concentration in the literature vary widely from submicromolar basal levels to several micromolar or higher with activation (32). There is less data reported in terms of the IP₃ concentrations in tissues. Several micromolar of IP₃ or higher has been indicated which is higher concentration than the \( IC_{50} \) for IP₃ binding (32).

Partial purification of a high affinity IP₃ binding protein from cerebellum has previously been reported by Theibert et al. (26) and Donie et al. (27). Both groups report binding specific for IP₃ \( (K_d = 1–10 \text{ nM}) \), but not for IP₄. After our manuscript was submitted, the purification of a high affinity IP₃ receptor and an IP₄ receptor was reported using an IP₃ affinity column (30). The IP₃ receptor is a heterotrimer of similar subunit composition (115, 105, and 50 kDa) to that reported here for the inositol polyphosphate receptor and appears to have similar binding characteristics. Competitive binding studies using \([\text{H}]\text{IP}_3\) indicate that a number of inositol polyphosphates (IP₄, IP₃, IP₅, and IP₁₄) bind to the receptor. The reported binding of IP₄ is high affinity (\( IC_{50} \sim 12 \text{ nM} \)) at low ionic strength. We find that binding is considerably weaker at isotonic salt concentration and that binding of IP₃ and IP₄ are not very different.

We have designated the receptor the inositol polyphosphate receptor to reflect its promiscuity of binding. Reconstitution studies with the inositol polyphosphate receptor preparation indicate an IP₃-modulated K⁺ channel. The role of the receptor and its further characterization, especially the channel gating and its relevance to function, remain to be determined.

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