# High-affinity inositol 1,3,4,5-tetrakisphosphate receptor from cerebellum: solubilization, partial purification and characterization

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Proteins which bind with high affinity Ins 1,3,4,5-$P_4$ or Ins 1,4,5-$P_3$ were solubilized from porcine cerebellar membranes. Both binding activities were separated by heparin-agarose chromatography. The Ins 1,3,4,5-$P_4$ receptor was partially purified with an approximately 1000-fold enrichment as compared to the membrane preparation. In the receptor-enriched preparation the Ins 1,3,4,5-$P_4$13 binding protein had an affinity ($K_d$) for Ins 1,3,4,5-$P_4$ of 4.6 nM. Ins 1,3,4,5,6-$P_5$ displaced [$^3$H]Ins 1,3,4,5-$P_4$binding with a comparable affinity. The Ins 1,3,4,5-$P_4$binding protein displayed high selectivity for Ins 1,3,4,5-$P_4$ over other inositolphosphates ($IC_{50}$ for Ins 1,4,5,6-$P_4$ 150 nM, for Ins-$P_6$ 1 $\mu$M and for Ins 1,3,4-$P_3$ 5 $\mu$M). Most importantly, Ins 1,4,5-$P_3$ did not displace [$^3$H]Ins 1,3,4,5-$P_4$binding at concentrations up to 10 $\mu$M. Binding of Ins 1,3,4,5-$P_4$ was maximal in the pH range between 4.5 and 6, was stable with $Ca^{2+}$ concentration varied from 1 nM to 1 mM, and was suppressed by heparin ($IC_{50}$ about 2 nM). The high affinity receptor for Ins 1,3,4,5-$P_4$ reported here, which is distinct from the Ins 1,4,5-$P_3$ receptor might allow to evaluate the possible functional role of Ins 1,3,4,5-$P_4$ in the cellular signal transduction.

Inositol 1,3,4,5-tetrakisphosphate; Inositol 1,4,5-trisphosphate; Inositolphosphate receptor; Heparin

## 1. INTRODUCTION

Inositol 1,4,5-trisphosphate (Ins 1,4,5-$P_3$; Ins-$P_3$) is generated in many different cell types as a second messenger after stimulation by hormones [1]. The role of Ins-$P_3$ has been clarified by physiological and biochemical experiments demonstrating that this reaction product of phospholipase C releases $Ca^{2+}$ from intracellular stores [1]. Conclusive evidence for the function of Ins-$P_3$ was given recently by inserting the isolated protein (Ins 1,4,5-$P_3$ receptor) into lipid vesicles. Ferris et al. [2] demonstrated in this reconstituted preparation that Ins-$P_3$ mediates $Ca^{2+}$ flux. Ins-$P_3$ is rapidly metabolized and thus inactivated by two routes [3]. Ins-$P_3$ is either dephosphorylated by Ins 1,4,5-$P_3$ 5-phosphatase to Ins 1,4-$P_2$, which has no effect on $Ca^{2+}$ levels [3]. Alternatively, Ins 1,4,5-$P_3$ 3-kinase yields the phosphorylation product inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5-$P_4$; Ins-$P_4$) [4]. Ins-$P_4$ has been suggested also to be involved in $Ca^{2+}$ homeostasis. Several still controversial hypotheses have been put forward suggesting mechanisms of action of Ins-$P_4$ such as $Ca^{2+}$ sequestration into internal pools [5], or regulation of the accessibility of different types of $Ca^{2+}$ pools [6].

Recently we have demonstrated the existence of high-affinity receptors for Ins-$P_4$ in cerebellar membranes [7]. The receptor showed a high selectivity for Ins 1,3,4,5-$P_4$ among several inositolphosphates tested. We have used the binding protein preparation from pig cerebellum to quantify cellular contents of Ins-$P_4$ in a neuronal cell line stimulated with the peptides bradykinin [7] or endothelin [8]. Our method greatly facilitates measurements of Ins-$P_4$ contents in tissues incubated with various hormones. Thus, evidence might be gathered for a possible role of Ins-$P_4$ in cellular signal transduction involving inositolphosphates.

However, a definitive proof for a function of Ins-$P_4$ can only be established by isolating the receptor for Ins-$P_4$ and demonstrating the functional properties of the protein. Here we report solubilization of the Ins-$P_4$ receptor from pig cerebellum and its partial purification. Furthermore, some characteristics of the receptor were investigated such as the influence of pH, of $Ca^{2+}$ concentration and of various polyglycanes on binding.

*Abbreviations:* Ins-$P_4$ (Ins 1,3,4,5-$P_4$), D-myo-inositol 1,3,4,5-$P_4$; Ins-$P_3$ (Ins 1,4,5-$P_3$), D-myo-inositol 1,4,5-$P_3$

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## 2. MATERIALS AND METHODS

### 2.1. Materials

[$^3$H]Ins(1,3,4,5)$P_4$ (spec. act. 755–1330 GBq/mmol), [$^3$H]-Ins(1,4,5)$P_3$ (spec. act. 1.2 TBq/mmol) were from Amersham, Braunschweig, FRG. Ins(1,3,4,5)$P_4$, Ins(1,3,4)$P_3$, Ins(1,4,5)$P_3$, potassium salts, Ins(1,2,3,4,5,6)$P_6$, sodium salt, were from Calbiochem, Frankfurt, FRG. Ins(1,4,5)$P_3$, Ins(1,3,4,5)$P_4$, Ins(1,4,5,6)$P_4$, Ins(1,3,4,5,6)$P_5$, ammonium salts were from Boehringer, Mannheim, FRG. Heparin (molecular weight 4000–6000), pentosan polysulfate, de-*N*-sulfated heparin, sodium salts, were from Sigma, Deisenhofen, FRG. Heparin-agarose, Amido Schwarz and Bio-Gel P-4 were from Biorad, Muñchen, FRG. Brij 58 (polyoxyethylene monocetyl ether)



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was from Serva, Heidelberg, FRG. All other chemicals were of analytical grade obtained from Merck, Darmstadt, FRG.

2.2. *Methods*

Membranes from pig cerebellum were prepared as described [7]. Briefly, the tissue was homogenized in buffer containing (mM) Tris-HCl 50, EDTA 1, mercaptoethanol 1, pH 7.7, at 4°C, using a Waring Blendor and centrifuged (30 min, 35 000 *g*). Subsequently the pellet was twice resuspended, homogenized and centrifuged. The final pellet, resuspended in buffer at a concentration of 20 to 30 mg/ml, was homogenized using a Potter-Elvejhem homogenizer and kept at −20°C.

For solubilization the membrane suspension was centrifuged (10 min, 27 000 *g*). The resulting membrane pellet was supplemented with solubilization buffer at a volume ratio of buffer to membranes of 5:1. The solubilization buffer contained (mM) Tris-HCl 50, EDTA 1, mercaptoethanol 5, pH 7.5, and 1.5 % (w/v) Brij 58. The mixture was gently stirred at 4°C for 1 h and centrifuged in a ultracentrifuge (1 h, 140 000 *g*). After centrifugation the supernatant without the vesicular layer was applied at a flow rate of 100 ml/h to a heparin-agarose column (35 ml, 4 cm diameter) equilibrated with buffer A (mM): Tris-HCl 50, EDTA 1, mercaptoethanol 1, pH 7.5, and 0.1 % (w/v) Brij 58. After washing the column with buffer A, proteins were eluted by stepwise increasing the NaCl concentration to 0.25, 0.4, 0.5 and 0.8 M. The peak of Ins-$P_4$ binding activity detected in the fractions of the 0.8 M NaCl eluate was pooled and used either for analysis or for re-chromatography on a second, smaller heparin-agarose column (1.5 ml, 1 cm diameter) which was eluted with buffer A containing NaCl at concentrations of 0.4 or 0.8 M.

Ins-$P_4$ binding activity was assayed in solubilized and purified preparations by spun-column chromatography. Samples were incubated for 20 min at 4°C with [$^3$H]Ins 1,3,4,5-$P_4$ (concentration 0.11–0.20 nM) in a total volume of 280 μl incubation buffer (in mM: EDTA 1, sodium acetate 25, pH 5.0, potassium phosphate 25, pH 5.0, mercaptoethanol 1, and 0.1% (w/v) Brij 58). An aliquot of the incubation mixture was applied to columns containing Biogel P-4 (1 ml packed resin) and centrifuged (2 min, 3500 *g*) to separate bound from unbound ligand. The void volume of the columns was supplemented with 3.5 ml scintillation liquid (Aqualuma plus, Baker). Also Ins-$P_3$ binding activity was determined by spun-column chromatography using the incubation buffer described previously [7]. Radioactivity was determined taking quenching into account by automatic correction with a quench standard curve (LKB, 1214 Rackbeta). Radioligand binding to membranes was measured as in [7].

Protein contents of samples of detergent-solubilized membranes or of detergent containing eluates from column chromatography were measured by the Amido Schwarz method described by Schaffner and Weissman [9] modified for Brij 58 containing samples.

## 3. RESULTS

Membranes from pig cerebellum were treated with detergent solutions for 1 h at 4°C. With 1.5% of the detergent Brij 58, a large part of Ins-$P_4$ binding activity could be recovered in the supernatant obtained after centrifugation at 140 000 *g*. Binding activity was measured using the incubation conditions, which had been found to be favourable for Ins-$P_4$ binding in membranes. However, at the concentration of 1.5 % Brij 58 used for solubilization the detergent reduced Ins-$P_4$ binding (data not shown). Treatment of solubilized membranes with trypsin abolished specific Ins-$P_4$ binding consistent with the notion that a protein represents the binding site.

Ins-$P_4$ binding to the high affinity receptor in cerebellar membranes is inhibited by heparin [7]. Thus, we tried to purify the receptor by affinity chromatography on heparin-agarose. The solubilized preparation was applied to heparin-agarose and eluted by stepwise increasing the NaCl concentration in the elution buffer. The data from a typical column run are shown in Table I. With NaCl concentrations of 0.25 M, 0.4 M, 0.5 M and 0.8 M, the lowest total Ins-$P_4$ binding activity (0.4 pmol) was detected in the 0.5 M fraction and the highest total binding activity of 6.7 pmol in the 0.8 M fraction (Table I).

Since also the Ins 1,4,5-$P_3$ receptor adheres to heparin-agarose [10] we measured Ins-$P_3$binding activity, in parallel, in the same fractions. Most of the Ins-$P_3$ binding was found in the fraction obtained with 0.4 M NaCl (total 7.8 pmol), whereas very little binding could be detected in the 0.8 M fraction (0.15 pmol). Binding activities were determined in samples dialyzed with buffer A containing no Brij 58. This desalting procedure avoided a possible interference of varying salt concentrations with inositolphosphate binding. The solubilized membranes displayed significantly more binding activity for Ins-$P_3$ than for Ins-$P_4$. In the fraction eluted

Table I

Elution profile of Ins 1,3,4,5-$P_4$ and Ins 1,4,5-$P_3$ binding activities from heparin-agarose chromatography. Membranes from pig cerebellum prepared as described [7] were suspended at 200 mg wet weight per ml buffer containing Brij 58 (1.5%). After solubilization and centrufigation, as described in Methods, the 140 000 × g supernatant was applied to a heparin-agarose column. Fractions of the eluates with increased absorbance at 280 nm and with inositolphosphate binding activity were pooled and dialyzed in buffer A devoid of Brij 58. Inositolphosphate binding activities shown here were determined with 0.19 nM [$^3$H]Ins 1,3,4,5-$P_4$ or [$^3$H]Ins 1,4,5-$P_3$ in the incubation mixture and analyzed by spun-column chromatography, as described in Methods. For measuring unspecific binding, 0.5 μM Ins 1,3,4,5-$P_4$ or μM Ins 1,4,5,-$P_3$ were added. Specific binding activities (= total binding - unspecific binding) of the dialyzed samples assayed in duplicate are given. Results are representative for 4 experiments.

| Fraction | Solubilized membranes | 0.25 M NaCl eluate | 0.4 M NaCl eluate | 0.5 M NaCl eluate | 0.8 M NaCl eluate |
|---|---|---|---|---|---|
| Total protein (mg) | 414 | 90.8 | 36.3 | 0.55 | 0.68 |
| Specific Ins-$P_4$ binding (fmol/mg protein) | 19.6 | 32.6 | 80.4 | 741 | 9818 |
| Specific Ins-$P_3$ binding (fmol/mg protein) | 57.7 | 35.8 | 217 | 327 | 602 |

with 0.8 M NaCl, specific Ins-$P_4$binding was 9.8 pmol/mg protein, whereas Ins-$P_3$ binding of only 0.22 pmol/mg protein could be detected. Thus binding activities for Ins-$P_4$and for Ins-$P_3$ were efficiently separated by heparin-agarose chromatography.

Using the fractions of the 0.8 M NaCl eluate with high binding activity, some of the properties of the Ins-$P_4$ receptor were determined. The dependence of Ins-$P_4$ binding on pH is demonstrated in Fig. 1. Specific binding is characterized by a bell-shaped curve with minimal binding below pH 4.25 and above pH 6.5. The maximum was reached between pH 4.5 and 6. Unspecific binding remained almost constant over the whole pH range tested.

In some experiments the Ins-$P_4$ binding activity recovered in the 0.8 M NaCl eluate from heparin-agarose chromatography was dialyzed and re-applied to heparin-agarose. The eluate obtained with 0.4 M NaCl was discarded since it contained very little Ins-$P_4$ binding activity. In the subsequent 0.8 M NaCl eluate, however, in a typical experiment binding activity for Ins-$P_4$, determined as described in Table I, was 12.5 pmol/mg protein. Whereas in the 0.8 M NaCl eluate from the first heparin-agarose column Ins-$P_3$ binding activity was still 10% of Ins-$P_4$ binding activity, the corresponding value after the second heparin-agarose column was only 4%. The ability of heparin to displace Ins-$P_4$ binding was tested in the receptor-enriched fraction obtained from the second affinity column (Fig. 2A). Binding was half-maximally inhibited by 15 $\mu$g/l heparin, a concentration of about 2.5 nM. De-*N*-sulfated heparin was about one order of magnitude less potent, whereas pentosan polysulfate displayed an intermediate affinity.

Fig. 2B shows binding characteristics of the Ins-$P_4$ receptor which has been partially purified by two heparin-agarose columns. [$^3$H]Ins-$P_4$ binding was displaced by unlabelled Ins-$P_4$ with half-maximal inhibition at 6 nM (Fig. 2B). Even after storage for 10 days at 4°C, the protein still showed the same binding characteristics. Scatchard analysis (Fig. 2B, inset) gives an affinity constant $K_d$ of 3.3 nM and a binding capacity of 1.6 nM, which corresponds to 300 pmol/mg protein. The mean value for $K_d$ was 4.6 ± 1.8 nM (n = 6). The specificity of the Ins 1,3,4,5-$P_4$ binding site was characterized by adding several inositololigophosphates. Ins 1,3,4,5,6-pentakisphospate displaced [$^3$H]Ins 1,3,4,5-$P_4$ binding nearly as potently as Ins 1,3,4,5-$P_4$. Ins 1,4,5,6-$P_4$ suppressed Ins 1,3,4,5-$P_4$ binding half-maximally at 150 nM. The other inositolphosphates tested displayed much lower affinity. Inositolhexakisphosphate and Ins 1,3,4-$P_3$ inhibited binding of Ins 1,3,4,5-$P_4$ half-maximally at about 1 and 5 $\mu$M, respectively. Ins 1,4,5-$P_3$, however, had no influence at concentrations up to 10 $\mu$M (data not shown). Also the influence of $Ca^{2+}$ on Ins 1,3,4,5-$P_4$ binding was tested by adjusting the $Ca^{2+}$ concentration with EGTA. $Ca^{2+}$

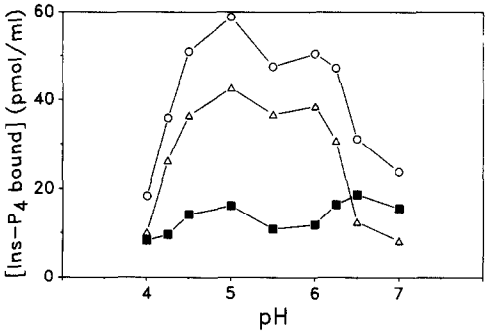



Fig. 1. Influence of pH on Ins 1,3,4,5-$P_4$ binding. Membrane proteins were solubilized, as described in Methods and separated by heparin-agarose chromatography. From the eluate with buffer A containing 0.8 M NaCl the fractions containing Ins-$P_4$ binding activity were combined and used for the experiment. For determining binding, the pH was adjusted to the various values indicated in the two buffering components (acetate and phosphate) of the incubation buffer separately. Specific binding (Δ) is obtained by subtracting unspecific binding (■) from total binding (○). Unspecific binding represents the amount of binding not displacable by 0.4 $\mu$M unlabelled Ins 1,3,4,5-$P_4$. [$^3$H]Ins-$P_4$ binding was measured by spun-column chromatography. Here and in Fig. 2 data representative for at least 2 further experiments with comparable results are shown.

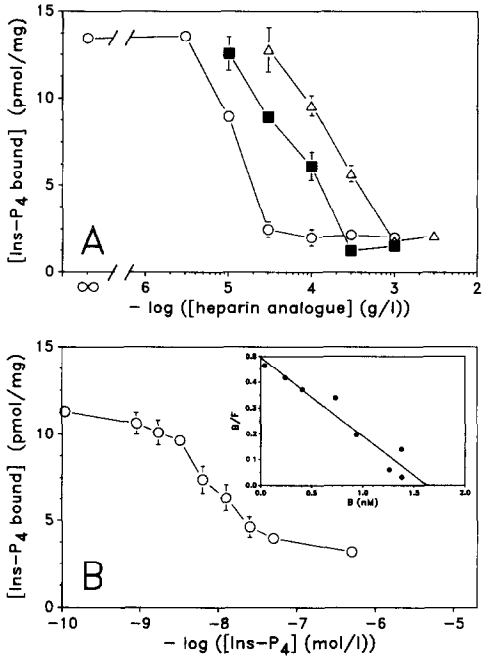



Fig. 2. Characteristics of the partially purified Ins 1,3,4,5-$P_4$ receptor. (A) Inhibition of Ins 1,3,4,5-$P_4$ binding by heparin and related analogues. (B) Affinity of the partially purified protein for Ins 1,3,4,5-$P_4$. In A varying amounts of heparin (○), de-*N*-sulfated heparin (Δ) or pentosan polysulfate (■) were present during the incubation. In (B) 0.14 nM [$^3$H]Ins 1,3,4,5-$P_4$ and varying concentrations of unlabelled Ins 1,3,4,5-$P_4$ were present. (Inset) Scatchard analysis of Ins 1,3,4,5-$P_4$ binding from the data shown in (B). For both experiments (A and B) fractions eluted from the second heparin-agarose column with buffer A containing 0.8 M NaCl were used. Values represent means obtained from duplicate incubations and error bars give the deviations. Protein concentration in the assay mixtures for determining binding activity was 5 $\mu$g/ml in A and in B.

did not affect binding of Ins 1,3,4,5-$P_4$ at a range of concentrations from about 1 nM to 1 mM (not illustrated).

## 4. DISCUSSION

We have identified binding sites for Ins 1,3,4,5-$P_4$ with an affinity of approximately 1 nM and a concentration of 0.22 pmol/ mg protein in membranes prepared from porcine cerebellum [7]. The density of Ins 1,4,5-$P_3$ binding activity with high affinity ($K_D$14 nM) was 7.3 pmol/ mg protein in these membranes from pig cerebellum, thus exceeding the Ins 1,3,4,5-$P_4$ receptor concentration by a factor of 30 to 40.

Both binding activities, for Ins-$P_3$ and for Ins-$P_4$, were solubilized with high efficiency by using the nonionic detergent Brij 58. The solubilized Ins-$P_4$ receptor displayed an affinity comparable to that of the receptor embedded in the membrane. The Ins-$P_3$ receptor has already been purified to homogeneity from rat cerebellum [10] and the primary structure of the Ins-$P_3$ receptor has been deduced recently from cDNA cloning analysis [11]. Like Ins-$P_3$ binding [12], also Ins-$P_4$ binding is suppressed by heparin [7]. Therefore the solubilized preparation was subjected to heparin-affinity chromatography. Both binding activities adhered to heparin-agarose and were eluted with a series of buffers with increasing ionic strength. The maximal Ins-$P_3$ binding activity was eluted at 0.4 M NaCl. This pattern is similar to the results obtained by Maeda et al. [13] and by Supattapone et al. [10] reporting that the high affinity Ins 1,4,5-$P_3$ receptor can be eluted from heparin-agarose by a buffer containing 0.5 M NaCl. Maximal Ins 1,3,4,5-$P_4$ binding, however, was found in the fraction eluted with 0.8 M NaCl.

Thus, Ins-$P_3$ and Ins-$P_4$ binding activities can be largely separated by heparin-agarose chromatography. After a second heparin-agarose column we obtained a fraction with a substantial concentration of the high affinity Ins-$P_4$ receptor (300 pmol/mg protein). This gives an approximately 1000-fold purification of Ins-$P_4$ binding activity in comparison with the membrane preparation (0.2 pmol/mg protein). In the final fraction obtained after the second heparin-agarose column, specific Ins-$P_3$ binding was very low amounting to about 4% of Ins-$P_4$ binding activity. During preparation of the present manuscript Theibert et al. [14] reported the solubilization of an Ins-$P_4$ binding protein from rat cerebellum. However, Ins-$P_4$ and Ins-$P_3$ binding proteins could not be separated by heparin chromatography.

The characteristics of Ins-$P_4$ binding activity which we found after purification resemble those detected in the membrane preparation. The selectivity for the various inositolphosphates was similar apart from the fact that Ins 1,3,4,5,6-$P_5$ inhibited [$^3$H]Ins 1,3,4,5-$P_4$ binding with an $IC_{50}$ close to that for non-radioactive Ins 1,3,4,5-$P_4$. The $IC_{50}$ value for Ins 1,4,5,6-$P_4$ was 150 nM, for Ins 1,3,4-$P_3$ 5 $\mu$M and for Ins-$P_6$ 1 $\mu$M. The fact that Ins 1,4,5-$P_3$ showed no inhibition up to 10 $\mu$M reveals most clearly that the protein isolated is distinct from the Ins 1,4,5-$P_3$ receptor.

Some of the properties of the Ins-$P_4$ binding activity were determined: variation of pH showed that Ins-$P_4$ binding was maximal in the range between 4.5 and 6. Ins-$P_3$ binding to its high affinity receptor, however, showed a different pH dependency. Ins-$P_3$ binding increased with raising the pH above 5 and reached a maximal value at a pH of about 8.5 [12]. $Ca^{2+}$ did not influence Ins-$P_4$ binding at a range of concentrations from nano- to millimolar, similarly to the lack of an effect of $Ca^{2+}$ on binding of Ins-$P_3$ to the purified Ins-$P_3$ receptor [10].

A remarkable property of Ins-$P_4$ binding activity is the inhibition by heparin and related polyglycans. Heparin suppresses high-affinity Ins-$P_3$ binding [12,15] and blocks Ins-$P_3$-induced $Ca^{2+}$ release [16,17]. These results have been exploited frequently for using heparin as an experimental tool in the following context: disappearance of a cellular effect after injection of heparin was taken as evidence that Ins 1,4,5-$P_3$ was involved in the activation process [18]. The similarly high affinity of heparin for the Ins 1,3,4,5-$P_4$ receptor shown here, however, warrants caution in interpreting such results using heparin. They might as well imply that Ins 1,3,4,5-$P_4$ is involved.

Finally, the Ins-$P_4$ receptor preparation of porcine cerebellum solubilized and partially purified provides the possibility for isolating the receptor and thus determining the molecular properties of the binding protein. Availability of the Ins-$P_4$ receptor should allow testing the various hypotheses concerning the putative physiological role of Ins 1,3,4,5-$P_4$ in cellular signal transduction.

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