A high-affinity inositol 1,3,4,5-tetrakisphosphate receptor protein from brain is specifically labelled by a newly synthesized photoaffinity analogue, *N*-(4-azidosalicyl)aminoethanol(1)-1-phospho-D-*myo*-inositol 3,4,5-trisphosphate

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A photolabile arylazido analogue of $\text{Ins}(1,3,4,5)\text{P}_4$ selectively substituted at the 1-phosphate group was synthesized by coupling 2-aminoethanol(1)-1-phospho-D-*myo*-inositol 4,5-bisphosphate with *N*-hydroxysuccinimidyl-4-azidosalicylic acid [Schäfer, Nehls-Sahabandu, Grabowsky, Dehlinger-Kremer, Schulz & Mayr (1990) *Biochem. J.* **272**, 817–825] and subsequently phosphorylating the product by bovine brain $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase. The product, *N*-(4-azidosalicyl)-aminoethanol(1)-1-phospho-D-*myo*-inositol 3,4,5-trisphosphate [$\text{AsaIns}(1,3,4,5)\text{P}_4$] was radioiodinated and purified by anion-exchange chromatography. $\text{AsaIns}(1,3,4,5)\text{P}_4$ bound to a high-affinity $\text{Ins}(1,3,4,5)\text{P}_4$ receptor from pig cerebellum with an affinity only 3-fold lower than that of $\text{Ins}(1,3,4,5)\text{P}_4$. Photoirradiation of ^{125}I - $\text{AsaIns}(1,3,4,5)\text{P}_4$ in the presence of the receptor preparation revealed that the radioactive label was specifically associated with a protein band of apparent molecular mass 42 kDa, which Donié & Reiser [(1991) *Biochem. J.* **275**, 453–457] had previously tentatively assigned to the $\text{Ins}(1,3,4,5)\text{P}_4$ receptor protein. The radioactive label was displaced from the receptor when the binding reaction with ^{125}I - $\text{AsaIns}(1,3,4,5)\text{P}_4$ was carried out in the presence of $5\text{ }\mu\text{M}$ - $\text{Ins}(1,3,4,5)\text{P}_4$.

INTRODUCTION

The second messenger $\text{Ins}(1,4,5)\text{P}_3$ [1] triggers the release of Ca^{2+} from internal stores [2] and possibly opens Ca^{2+} channels in the plasma membrane [3,4]. The $\text{Ins}(1,4,5)\text{P}_3$ -specific receptor [5] apparently consists of four identical 260 kDa polypeptides. Their primary structure has been deduced from mouse [6] and rat brain [7] cDNA. The Ca^{2+} -mobilizing function of $\text{Ins}(1,4,5)\text{P}_3$ has been confirmed by the finding that the purified $\text{Ins}(1,4,5)\text{P}_3$ receptor reconstituted into liposomes and challenged by InsP_3 mediated an enhanced $^{45}\text{Ca}^{2+}$ flux [8]. The InsP_3 receptor inserted into planar lipid bilayers exhibited ion-channel activity [9]. This indicates that the Ca^{2+} -release channel of the endoplasmic reticulum may be solely made up from the $\text{Ins}(1,4,5)\text{P}_3$ receptor protein.

The involvement of $\text{Ins}(1,3,4,5)\text{P}_4$, a phosphorylation product of $\text{Ins}(1,4,5)\text{P}_3$ [10,11], in cellular Ca^{2+} regulation is not yet clearly understood. Some data indicate that $\text{Ins}(1,3,4,5)\text{P}_4$ induces re-uptake of Ca^{2+} into internal stores [12]. Alternatively, $\text{Ins}(1,3,4,5)\text{P}_4$ has been suggested to control, in concert with $\text{Ins}(1,4,5)\text{P}_3$, the Ca^{2+} entry from the extracellular compartment [13,14]. Thus $\text{Ins}(1,3,4,5)\text{P}_4$ could be involved in the 'quantal' release of Ca^{2+} (see discussion in ref. [15]) induced by $\text{Ins}(1,4,5)\text{P}_3$ [14,16]. However, the site of action of $\text{Ins}(1,3,4,5)\text{P}_4$ is still unclear. Recently, Donié *et al.* [17] reported the solubilization and purification of a high-affinity $\text{Ins}(1,3,4,5)\text{P}_4$ receptor from pig cerebellum. The receptor was identified as a protein with an apparent molecular mass of 42 kDa by SDS/PAGE [18]. From rat brain Theibert *et al.* [19] have isolated two separate fractions

with high-affinity $\text{Ins}(1,3,4,5)\text{P}_4$ -binding activities containing proteins with apparent molecular masses of 174 and 84 kDa and 182 and 123 kDa respectively. However, a receptor protein was not yet identified.

Functional identification of the 42 kDa protein as the high-affinity $\text{Ins}(1,3,4,5)\text{P}_4$ receptor was not yet possible, since no covalently binding ligand for the receptor was available. Therefore an attempt was made to synthesize a photoaffinity analogue of $\text{Ins}(1,3,4,5)\text{P}_4$, by a method similar to that already used successfully for synthesis of a photoaffinity analogue of $\text{Ins}(1,4,5)\text{P}_3$ [20].

Here we report synthesis of a photolabile $\text{Ins}(1,3,4,5)\text{P}_4$ analogue, *N*-(4-azidosalicyl)aminoethanol(1)-1-phospho-D-*myo*-inositol 3,4,5-trisphosphate [$\text{AsaIns}(1,3,4,5)\text{P}_4$]. AsaInsP_4 displaces [^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ bound to the high-affinity $\text{Ins}(1,3,4,5)\text{P}_4$ receptor protein purified from pig brain [18]. Furthermore, ^{125}I - $\text{AsaIns}(1,3,4,5)\text{P}_4$ specifically labels the 42 kDa protein, corroborating the function of this protein as an $\text{Ins}(1,3,4,5)\text{P}_4$ receptor.

MATERIALS AND METHODS

Materials

[^3H] $\text{Ins}(1,3,4,5)\text{P}_4$ (sp. radioactivity 1020–1330 GBq/mmol) and Na^{125}I were from Amersham Buchler (Braunschweig, Germany). $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ (potassium salts) and $\text{Ins}(1,2,3,4,5,6)\text{P}_6$ (sodium salt) were from Calbiochem (Frankfurt, Germany). $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$ (ammonium salts) were from Boehringer (Mannheim, Germany). Other inositol phosphate isomers have been purified and struc-

Abbreviations used: AsaInsP_4 , [$\text{AsaIns}(1,3,4,5)\text{P}_4$], *N*-(4-azidosalicyl)aminoethanol(1)-1-phospho-D-*myo*-inositol 3,4,5-trisphosphate; AsaInsP_3 , [$\text{AsaIns}(1,4,5)\text{P}_3$], *N*-(4-azidosalicyl)aminoethanol(1)-1-phospho-D-*myo*-inositol 4,5-bisphosphate; $\text{GroPIns}(4,5)\text{P}_2$, α -glycero(3)-1-phospho-D-*myo*-inositol 4,5-bisphosphate; IC_{50} , half-maximal inhibitory concentration.

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turally identified by n.m.r. spectroscopy in G.W.M.'s laboratory [21–23]. Affi-Gel Heparin (heparin–agarose), Amido Black, Bio-Gel HT (hydroxyapatite) and Bio-Gel P4 were from Bio-Rad (München, Germany.). CM-cellulose was from Whatman (Maidstone, Kent, U.K.). Brij 58 (polyoxyethylene monooctyl ether), calmodulin and chemicals for electrophoresis were from Serva (Heidelberg, Germany). Mono Q resin (Mono Q 5/5 columns), Q-Sepharose and molecular-mass standards were from Pharmacia (Freiburg, Germany). Sources of chemicals for the synthesis of AsaIns(1,3,4,5) P_4 were described in [20]. All other chemicals were of analytical grade.

Methods

Synthesis of AsaIns(1,3,4,5) P_4 . All reactions were performed in the dark. A 50 nmol portion of AsaIns(1,4,5) P_3 , synthesized and purified as described in [20], was phosphorylated in a volume of 300 μ l at 30 °C. The phosphorylation mixture contained 150 μ M-CaCl₂, 0.2 μ M-calmodulin, 1.5 mM-ATP, 3 mM-MgCl₂, Ins-(1,4,5) P_3 3-kinase (4 nmol/ml per min) and 10 mM-Tris/HCl, adjusted to pH 7.5. Ins(1,4,5) P_3 3-kinase from bovine brain was purified as described in [20]. After 30 (60) min of incubation, 90 % (97 %) of AsaIns(1,4,5) P_3 was converted into AsaIns-(1,3,4,5) P_4 , as assessed by h.p.l.c. analysis with u.v.-absorbance monitoring (see below). Therefore the enzymic reaction was quenched after 60 min by adding 200 μ l of ice-cold ethanol and 550 μ l of ice-cold water. A 1 ml portion of the mixture was injected on to a 5 mm $\times$ 25 mm Mono Q column equilibrated with 10 mM-ammonium acetate, pH 5.5 (eluent A). The reaction tube and the injection vessel were washed with 1.05 ml of 10 % (v/v) ethanol, which was also injected. The column run without a cellulose/glass-fibre prefilter [23] was eluted at a flow rate of 0.8 ml/min with the following gradient to 2.0 M-ammonium acetate, pH 5.5 (eluent B): 0–4 min, eluent A; 4–8 min, 0–40 % B; 8–28 min, 40–100 % B; 28–35 min, 100 % B. Fractions (1.2 ml) were collected, and 20 μ l of each fraction was hydrolysed for 30 min in 1 M-H₂SO₄ at 160 °C for phosphate analysis. To assess the purity of phosphate-containing fractions and to evaluate the degree of substrate conversion, 50 μ l samples from the column fractions and 15 μ l samples from the enzyme reaction mixture were analysed by h.p.l.c. by using the same column and elution protocol but with continuous monitoring of the u.v. absorbance (268 nm) of the effluent. Fractions 20–22 from the column run containing AsaIns(1,3,4,5) P_4 were pooled and freeze-dried in a thin film for 24 h in order to remove all the carrier salt. The vessel was rinsed with 2 ml of 20 % ethanol and briefly freeze-dried to recover the compound (15 nmol), which was dissolved in 300 μ l of water and stored at –20 °C.

Radioiodination of AsaIns(1,3,4,5) P_4 . This was performed similarly to the procedure described in [20]: 1.5 nmol of AsaIns(1,3,4,5) P_4 in 30 μ l of water was added to 90 μ l of incubation buffer containing ammonium acetate (25 mM), pH 8.0, ethanol (20 %), dimethylformamide (20 %, v/v), supplemented with 1.5 nmol of Na¹²⁵I (19 MBq) and one Iodo-bead, which had been washed twice in the same buffer. After a 4 h incubation period in the dark at room temperature, the Iodo-bead was removed and washed with incubation buffer (2 $\times$ 200 μ l). The wash solution was combined with the incubation mixture, 100 μ l of 0.1 M-NaI in incubation buffer was added, and the resulting mixture was loaded on to a 60 μ l column of Q-Sepharose (in a pipette tip), which was eluted stepwise with 10 $\times$ 80 μ l of 100 mM-ammonium acetate, pH 5.0, in 20 % ethanol (eluting [¹²⁵I]iodide), 4 $\times$ 80 μ l of 200 mM-ammonium acetate, pH 5.0, in 20 % ethanol, 8 $\times$ 80 μ l of 1.0 M-ammonium acetate, pH 5.0, in 50 % ethanol (eluting AsaIns P_3) and 4 $\times$ 80 μ l of 2.0 M-ammonium acetate, pH 5.0, in 50 % ethanol (eluting AsaIns P_4). Fractions containing ¹²⁵I-AsaIns(1,3,4,5) P_4 were pooled and freeze-dried to remove

carrier salt and ethanol. About 0.6 nmol of iodinated product with a specific radioactivity of 313 Ci/mmol was recovered. Analysis of the product by t.l.c. and h.p.l.c. [20,24] revealed respectively a single radioactive spot and one radioactive peak only.

Solubilization and purification of the Ins(1,3,4,5) P_4 receptor. Preparation of membranes from pig cerebella and solubilization were carried out as described [17], with some minor modifications to improve the yield of active receptor. In some cases the proteinase inhibitors phenylmethanesulphonyl fluoride (100 μ M), leupeptin (5 μ M) and chymostatin (5 μ M) were added to all the buffers used for membrane preparation [17]. In these experiments the proteinase inhibitors were also present at the same concentrations in the solutions employed for the following solubilization and purification.

For solubilization, a membrane pellet was mixed with solubilization buffer at a ratio of 4–5 ml of buffer to 1 g of membranes (wet wt.). The solubilization buffer contained Tris/HCl (50 mM), EDTA (1 mM), NaCl (400 mM), mercaptoethanol (5 mM), pH 7.5, and Brij 58 (1.5 %, w/v). The mixture was stirred at 4 °C for 1 h, sonicated for 2 $\times$ 30 s (Branson sonifier, setting 5, 50 % duty cycle) and centrifuged (1 h, 100 000 g). The supernatant was dialysed in buffer A, and was applied to a CM-cellulose column (3 cm $\times$ 5 cm) equilibrated with buffer A. Buffer A contained Tris/HCl (50 mM), EDTA (1 mM), mercaptoethanol (1 mM), pH 7.5, and Brij 58 (0.1 %). After washing with buffer A, proteins were eluted by increasing the NaCl concentration stepwise to 50 mM and 0.2 M. The fractions with Ins P_4 -binding activity eluted with 0.2 M-NaCl were pooled, dialysed against buffer A and applied to a heparin–agarose column (2 cm $\times$ 1.7 cm) equilibrated with buffer A. This column was eluted by increasing the NaCl concentration discontinuously to 0.4 M-, 0.5 M- and 0.8 M-NaCl. The fractions of the 0.8 M-NaCl eluate with Ins P_4 -binding activity were pooled, dialysed in buffer B and subjected to hydroxyapatite chromatography (1 cm $\times$ 0.5 cm column). Buffer B contained KH₂PO₄/K₂HPO₄ (25 mM), EDTA (1 mM), mercaptoethanol (1 mM), pH 7.5, and Brij 58 (0.1 %). After washing with buffer B, the fractions eluted with buffer B containing 1 M-NaCl showing Ins P_4 -binding activity were pooled and dialysed in buffer A. Ins P_4 -binding activity was assayed in solubilized and purified preparations by spun-column chromatography [17].

Photoaffinity labelling with ¹²⁵I-AsaIns P_4 . Samples of column eluates were incubated for 20 min in the dark in buffer containing: sodium acetate (25 mM), pH 5.0, potassium phosphate (25 mM), pH 5.0, EDTA (1 mM) and Brij 58 (0.1 %), and ¹²⁵I-AsaIns P_4 (20 nM). The reactions were carried out in a 48-well culture plate (Costar) kept on ice. Photolysis was achieved by a subsequent 20 min irradiation at 4 °C of the continuously stirred samples. We used u.v. light from a Philips HPK lamp (125 W, distance 15 cm) filtered through a filter of a saturated CuSO₄ solution (10 mm light-path). After irradiation, the proteins were precipitated by adding 4 vol. of methanol/acetone (1:1, v/v) and incubating for 10 min at 37 °C. The mixture was centrifuged at 10 000 g for 10 min and washed. For electrophoresis the samples were incubated overnight in buffer [final concns.: Tris (125 mM), pH 6.8, urea (4 M), mercaptoethanol (5 %), SDS (2 %) and Bromophenol Blue (0.002 %)] at 4 °C. Discontinuous SDS/PAGE was carried out as described by Laemmli [25]. The gel was stained with Coomassie Brilliant Blue R250 or silver [26], dried and placed on Hyperfilm MP (Amersham). Films were exposed at –70 °C for 2–20 h.

Protein contents of samples of detergent-solubilized membranes or of detergent-containing eluates from column chromatography were measured, after precipitation with trichloroacetic acid, by using Amido Black as described by Schaffner & Weissman [27], modified for samples containing Brij 58 [17].

Fig. 1. H.p.l.c. analysis of the product of AsaIns(1,4,5) P_3 phosphorylation by Ins(1,4,5) P_3 3-kinase

U.v. analysis of chromatograms of AsaIns(1,4,5) P_3 and AsaIns(1,3,4,5) P_4 . In trace A 5 nmol of purified AsaIns(1,4,5) P_3 and in trace B the same amount taken from the phosphorylation mixture after 60 min of incubation were applied. The retention times for AsaIns(1,4,5) P_3 and AsaIns(1,3,4,5) P_4 were 18.7 and 24.0 min respectively. (b) H.p.l.c. analysis with metal dye detection [24] of AsaIns P_3 , AsaIns P_4 and an alkali hydrolysate of AsaIns P_4 . A 5 mm $\times$ 100 mm column of Mono Q was operated with the strongly acidic elution system described in [24] at an eluent flow rate of 1.5 ml/min and a flow of the post-column reagent of 0.75 ml/min. An upward-concave gradient from 0 to 0.5 M-HCl with a duration of 35 min, similar in shape to that defined in [24], was employed. Trace A represents a mixture of inositol phosphates prepared from partially hydrolysed phytic acid [24], which was supplemented with 0.35 nmol of Ins(1,3,4,5) P_4 (marked by the broken line). The next peak to be eluted in trace A corresponds to 0.77 nmol of Ins(2,3,4,5) P_4 . This isomer of Ins P_4 is co-eluted with the small shoulder of the major peak in trace D (below). In trace B, 3.3 nmol of AsaIns(1,4,5) P_3 was analysed. From the phosphorylation mixture a corresponding amount taken after 60 min of incubation was injected without further treatment (trace C) or after boiling for 30 min in 1 M-KOH and neutralization with HCl (trace D, 60% of the sample taken). In traces C and D the early-eluted material represents ATP and ADP. In trace D only Ins(1,3,4,5) P_4 , Ins(2,3,4,5) P_4 (generated by alkali hydrolysis, besides the former product) and a small amount of Ins P_3 eluted at the position of Ins(1,4,5) P_3 were found. Details of the phosphorylation reaction and chromatography are described in the Materials and methods section.

RESULTS

Synthesis of AsaIns(1,3,4,5) P_4

The 1-phosphate group of Ins(1,4,5) P_3 is apparently not essential for Ins(1,4,5) P_3 to be accepted as a substrate of Ins(1,4,5) P_3 3-kinase. Therefore both GroPIns(4,5) P_2 and arylazido analogues of Ins(1,4,5) P_3 substituted at the 1-phosphate [20] are phosphorylated by this enzyme at position 3. An enzymic/synthetic approach was used to obtain an arylazido analogue of Ins(1,3,4,5) P_4 with a 1-phosphate substitution, which is structurally related to AsaIns(1,4,5) P_3 . AsaIns P_3 is a relatively stable photoaffinity analogue which can be readily radioiodinated, purified within some hours and used as an affinity label with high specific radioactivity, suitable to detect non-abundant specific high-affinity receptors of Ins(1,4,5) P_3 [20]. AsaIns(1,4,5) P_3 was enzymically converted into AsaIns(1,3,4,5) P_4 (structure shown in Fig. 2b, insert) by Ins(1,4,5) P_3 3-kinase from brain, purified by h.p.l.c. to homogeneity and subsequently radioiodinated. The enzyme almost completely converted AsaIns(1,4,5) P_3 into AsaIns(1,3,4,5) P_4 when high substrate concentrations were employed (Fig. 1). However, the final yield of pure product was greatly decreased, owing to losses by hydrophobic adsorption during purification of small amounts in the nanomole range.

An alternative but related synthetic route to AsaIns(1,3,4,5) P_4 implies phosphorylation of GroPIns(4,5) P_2 to GroPIns(3,4,5) P_3 with Ins(1,4,5) P_3 3-kinase, subsequent conversion into 2-aminoethanol(1)-1-phospho-D-*myo*-inositol 3,4,5-trisphosphate, and coupling with *N*-hydroxysuccinimidyl-4-azidosalicylic acid as described in [20]. This method was also employed successfully (results not shown), but the purification of nanomole amounts of the coupling product caused comparable recovery problems.

When purified AsaIns P_4 was alkali-hydrolysed [20] and analysed by h.p.l.c. with metal-dye detection [24], only the products expected for a derivative of Ins(1,3,4,5) P_4 with a 1-phosphodiester group were obtained: 80% Ins(1,3,4,5) P_4 , 17% Ins(2,3,4,5) P_4 and less than 3% Ins(1,4,5) P_3 (Fig. 1b). Thus the isomeric homogeneity of the synthetic product was established unambiguously. Since radioiodinated AsaIns(1,3,4,5) P_4 was relatively unstable, owing to radiation damage, only samples of AsaIns(1,3,4,5) P_4 were iodinated before experimental use. By using equimolar amounts of AsaIns(1,3,4,5) P_4 and [125 I]iodide, a product with specific radioactivity of some hundred Ci/mmol was obtained, which is necessary for specific labelling of high-affinity Ins P_4 -binding proteins (see below).

The Ins(1,3,4,5) P_4 receptor from cerebellum

Previously we have determined the binding specificity of the

Fig. 2. Specificity of the InsP_4 receptor purified from cerebellar membranes

(a) Displacement of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ binding by various inositol phosphates. The receptor protein was purified by CM-cellulose, heparin-agarose and hydroxyapatite chromatography as described in the Materials and methods section. Binding was determined as described in [17], with $0.8 \mu\text{g}$ of protein in the assay. The inositol phosphates tested were: $\circ$, $\text{Ins}(1,3,4,5)\text{P}_4$; $\blacksquare$, $\text{Ins}(1,2,5,6)\text{P}_4$; $\square$, $\text{Ins}(1,2,3,4,5)\text{P}_5$; $\triangle$, $\text{Ins}(1,3,4,5,6)\text{P}_5$; $\bullet$, $\text{Ins}(1,3,4,6)\text{P}_4$; $\blacktriangledown$, $\text{Ins}(3,4,5,6)\text{P}_4$; $\blacktriangledown$, $\text{Ins}(1,4,5,6)\text{P}_4$; $\diamond$, $\text{Ins}(1,2,3,4,6)\text{P}_5$; ∇ , $\text{Ins}(1,2,4,5,6)\text{P}_5$. (b) Binding of the azido analogue of InsP_4 , AsaInsP_4 , to the purified receptor preparation: displacement of $[^3\text{H}]\text{InsP}_4$ binding by InsP_4 ($\circ$) or by the photoaffinity analogue AsaInsP_4 ($\bullet$). Binding was determined in duplicates (deviations shown by error bars when exceeding the size of the symbols used) with a sample from the eluate obtained from the hydroxyapatite column. In (a) and (b) 100% specific binding (B_0) in the absence of inhibitors corresponds to 15 pmol of $\text{Ins}(1,3,4,5)\text{P}_4$ bound/mg of protein at 0.1 nM-InsP_4 . Comparable results were obtained in two (a) and one (b) further experiments. The insert shows the structural formula of $\text{AsaIns}(1,3,4,5)\text{P}_4$.

InsP_4 receptor [17,28]. Those studies were extended here. We tested the influence of additional inositol oligophosphates on InsP_4 binding, firstly to the receptor in the membrane and secondly also to the solubilized receptor. Displacement curves obtained with membranes showed that all the inositol phosphates had a much lower affinity than $\text{Ins}(1,3,4,5)\text{P}_4$. The half-maximally inhibitory concentrations (IC_{50}) of the tetrakisphosphates $\text{Ins}(1,2,5,6)\text{P}_4$ and $\text{Ins}(1,3,4,6)\text{P}_4$ and of the pentakisphosphates $\text{Ins}(1,2,3,4,5)\text{P}_5$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$ were 40–120 times that of $\text{Ins}(1,3,4,5)\text{P}_4$ (results not shown). An analogous experiment carried out with the solubilized and purified receptor is presented in Fig. 2(a). In the experiment depicted, the affinity constant for $\text{Ins}(1,3,4,5)\text{P}_4$ obtained from Scatchard analysis was 12 nM. Apart from $\text{Ins}(1,3,4,5,6)\text{P}_5$, all the inositol phosphates investigated

suppressed binding of $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ half-maximally in a narrow concentration range between 600 nM and $2 \mu\text{M}$.

In similar experiments, the effect of the newly synthesized photoaffinity analogue, $\text{AsaIns}(1,3,4,5)\text{P}_4$, on $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ binding was studied. With the purified receptor preparation, AsaInsP_4 inhibited $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ binding with an apparent affinity only about 3 times lower than that of $\text{Ins}(1,3,4,5)\text{P}_4$ (Fig. 2b). A competitive interaction of AsaInsP_4 and InsP_4 at the InsP_4 -binding site was further demonstrated by experiments testing the binding of $^{125}\text{I-AsaInsP}_4$ performed in darkness without subsequent irradiation. The amount of the iodinated photoaffinity analogue $^{125}\text{I-AsaInsP}_4$ bound to the purified receptor was suppressed by 80% by $0.5 \mu\text{M-InsP}_4$ but was not affected by $\text{Ins}(1,4,5)\text{P}_3$ at $2 \mu\text{M}$ concentration (results not shown). Thus we can conclude that AsaInsP_4 specifically interacts with the high-affinity InsP_4 receptor.

Figs. 3(a) and 3(b) (lanes 7 and 8) demonstrate that, among the proteins obtained after the hydroxyapatite chromatography, one single protein band specifically binds $^{125}\text{I-AsaInsP}_4$. For photoaffinity-labelling experiments, the samples were incubated with $20 \text{ nM-}^{125}\text{I-AsaInsP}_4$ to obtain equilibrium binding of the ligand, then irradiated with u.v. light and finally assayed by SDS/PAGE and silver-stained (Fig. 3a). The proteins with the covalently bound ligand could be detected by autoradiography (Fig. 3b). In the fraction from the hydroxyapatite column, which contained the purified receptor, only one protein band with apparent molecular mass 42 kDa is strongly labelled in the absence of non-radioactive InsP_4 (Fig. 3, lane 7). A comparatively negligible amount of binding is associated with a second protein band of 32 kDa. Fig. 3 (lane 8) shows the result from an incubation carried out in the presence of a high concentration ($5 \mu\text{M}$) of non-radioactive InsP_4 : the 42 kDa protein band had incorporated only a small residual amount of $^{125}\text{I-AsaInsP}_4$, and most of the binding was found associated with the small-molecular-mass band. The optical-density scan of an autoradiogram (Fig. 3c) quantifies this displacement of $^{125}\text{I-AsaInsP}_4$. With a receptor preparation derived after hydroxyapatite chromatography, 84% of the bound label was attached to the 42 kDa protein band in the absence of non-radioactive InsP_4 (Fig. 3c, continuous trace). In the presence of $5 \mu\text{M-InsP}_4$ only 20% of the label was bound to the 42 kDa band, and 60% to the 32 kDa protein band (Fig. 3c, broken trace).

The 32 kDa protein band binds AsaInsP_4 non-specifically with low affinity. This unspecific binding is very small in lane 7, because the binding reaction was carried out with a concentration of receptor-binding sites exceeding the concentration of $^{125}\text{I-AsaInsP}_4$ by approx. 20-fold. Thus the concentration of the free ligand was decreased to such an extent that the binding to the low-affinity sites became negligible. Only at the high concentration of non-radioactive InsP_4 (lane 8) was sufficient $^{125}\text{I-AsaInsP}_4$ displaced from the 42 kDa protein and thus available for the unspecific binding sites of the 32 kDa protein and of the low-molecular-mass proteins running with the front in the gel electrophoresis.

Furthermore, we determined the binding of $^{125}\text{I-AsaInsP}_4$ in some of the fractions obtained from the CM-cellulose and the heparin-agarose column in order to find out whether other proteins specifically bind InsP_4 . In the eluate obtained after the first purification step (CM-cellulose), which still contained many different proteins ([18] and Fig. 3d), a range of proteins bound $^{125}\text{I-AsaInsP}_4$ (note that the gel shown in Fig. 3d has been stained with Coomassie Blue only, because with these samples after silver staining individual protein bands could no longer be resolved). However, bound $^{125}\text{I-AsaInsP}_4$ could not be displaced by InsP_4 suggesting only unspecific binding (Fig. 3d). In the fractions obtained both from the CM-cellulose (Fig. 3d) and

Fig. 3. Photoaffinity labelling with ^{125}I -AsaIns P_4 and SDS/PAGE analysis of fractions obtained during purification of the high-affinity Ins P_4 receptor, demonstrating that only the 42 kDa protein band specifically binds ^{125}I -AsaIns P_4 , which can be displaced by Ins P_4 .

Incubations for binding and subsequent photoaffinity reactions were carried out as described in the Materials and methods section. For binding reactions the mixtures used for lanes 1, 3, 5 and 7 in (a) and (b), and for lane 1 in (c) and (d), were incubated with ^{125}I -AsaIns P_4 in the absence of unlabelled Ins P_4 (marked '—'), and those used for lanes 2, 4, 6 and 8 in (a) and (b) and for lane 2 in (c) and (d) in the presence of $5\ \mu\text{M}$ -Ins P_4 (marked '+'). (a) SDS/PAGE analysis (10%-polyacrylamide running gel, 3% stacking gel), stained with Coomassie Brilliant Blue R250 and afterwards with silver. Lanes 1–6 contain samples from the heparin-agarose column: lanes 1 and 2, $84\ \mu\text{g}$ of protein eluted by $0.4\ \text{M}$ -NaCl; lanes 3 and 4, $35\ \mu\text{g}$ of protein eluted by $0.5\ \text{M}$ -NaCl; lanes 5 and 6, $6.7\ \mu\text{g}$ of protein eluted by $0.8\ \text{M}$ -NaCl. Lanes 7 and 8 contain samples from the hydroxyapatite column: $8.2\ \mu\text{g}$ of protein eluted by $1\ \text{M}$ -NaCl. Positions of molecular-mass markers are indicated beside the gel: α -lactalbumin (14 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), phosphorylase *b* (94 kDa). For electrophoresis, proteins were precipitated by incubation with 4 vol. of methanol/acetone (1:1, v/v) for 10 min at 37°C , centrifuged at $10000\ g$ for 10 min and incubated overnight in sample buffer [final concns.: Tris (125 mM), pH 6.8, urea (4 M), mercaptoethanol (5%), SDS (2%) and Bromophenol Blue (0.002%)] under reducing conditions at 4°C . (b) Autoradiogram of the gel shown in (a). The position of the protein band stained specifically by ^{125}I -AsaIns P_4 in lane 7 is marked by a large arrow. In the presence of $5\ \mu\text{M}$ non-radioactive Ins P_4 the radioactive label was mainly bound to a protein marked by a small arrow (lane 8). Lanes 5 and 6 correspond to replicate incubations carried out with identical samples eluted by $0.8\ \text{M}$ -NaCl from the heparin-agarose column; however, the amount of radioactive ^{125}I -AsaIns P_4 during the incubation of the mixture used for lane 5 was incidentally lower than that employed in lane 6. Nevertheless the example is used for illustration, because all protein samples used for lanes 1–8 are derived from the same purification run. In the samples used for lanes 1, 2, 3, 4, 6, 7 and 8 the amount of radioactivity used was identical. The experiment with proteins from the heparin-agarose column run (lanes 1–6) was repeated twice with the same results. Results similar to those with the sample from the hydroxyapatite column shown in lanes 7 and 8 were obtained in five further experiments. (c) Densitometric scan of autoradiogram obtained with partially purified receptor eluted from the hydroxyapatite column. Samples with $9\ \mu\text{g}$ of protein from a hydroxyapatite column were incubated with ^{125}I -AsaIns P_4 in the absence (—) or presence (+) of $5\ \mu\text{M}$ -Ins P_4 , subjected to SDS/PAGE and subsequent autoradiography. The autoradiogram is shown in the left half of the diagram. The position of the 42 kDa protein is marked by the large arrow, and that of the 32 kDa protein by the small arrow. The film was scanned with a laser densitometer (Pharmacia gel scanner). The continuous and broken lines correspond to lanes 1 (—) and 2 (+) respectively. (d) Proteins obtained from CM-cellulose chromatography were analysed by SDS/PAGE (left half) and autoradiography (right half). Mixtures containing $45\ \mu\text{g}$ of protein eluted by $0.2\ \text{M}$ -NaCl from the CM-cellulose column (see the Materials and methods section) were incubated with ^{125}I -AsaIns P_4 in the absence (lane 1) or presence (lane 2) of $5\ \mu\text{M}$ -Ins P_4 . Further details were as for (a) and (b).

from the heparin-agarose column (Figs. 3a and 3b, lanes 1–6), unspecific binding of ^{125}I -AsaIns P_4 was mainly associated with the 46 kDa protein band and could not be suppressed by $5\ \mu\text{M}$ non-radioactive Ins P_4 (lanes marked with '+').

DISCUSSION

For the high-affinity Ins(1,4,5) P_3 receptor, structure-activity relationships have been established by determining receptor

binding and biological activity, i.e. Ca^{2+} release, of the various inositol phosphates. Studies on permeabilized cells have indicated that the vicinal phosphate groups at positions 4 and 5 are essential for Ca^{2+} release, and the monophosphate ester at position 1 only increases the affinity [29–31]. Recently, analogues of $\text{Ins}(1,4,5)\text{P}_3$ have been synthesized by Schäfer *et al.* [20] by selectively modifying the 1-phosphate position. The analogues with a photolabile group attached induced release of Ca^{2+} from endoplasmic reticulum and labelled three proteins of small molecular mass.

A similar strategy was used here for synthesis of an active photoaffinity analogue of $\text{Ins}(1,3,4,5)\text{P}_4$. Thereby the property of purified $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase to accept analogues of $\text{Ins}(1,4,5)\text{P}_3$ which are substituted at position 1 via a phosphodiester group as substrates was employed. The two analogues used here, $\text{AsaIns}(1,4,5)\text{P}_3$ and $\text{GroPIns}(4,5)\text{P}_2$, appear to be converted at concentrations above $30\text{ }\mu\text{M}$ with velocities and affinities comparable with those found with $\text{Ins}(1,4,5)\text{P}_3$. Biological activity of AsaInsP_4 cannot be measured, since the physiological function of $\text{Ins}(1,3,4,5)\text{P}_4$ has not yet been found. Binding experiments showed that the photoaffinity analogue displaced $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ from the receptor. With the purified receptor preparation, the affinity for AsaInsP_4 was only slightly lower than that for InsP_4 . Since, conversely, binding of $^{125}\text{I-AsaInsP}_4$ was also inhibited by InsP_4 , the two ligands AsaInsP_4 and InsP_4 compete for the same binding site. Thus modification of the 1-phosphate group of InsP_4 obviously only decreases the affinity of the ligand, but still allows interaction with the receptor.

We had already demonstrated the selectivity of the InsP_4 -binding site in cerebellar membranes [28]. $\text{Ins}(1,3,4,5,6)\text{P}_5$ showed the highest interfering affinity, but the IC_{50} ($0.2\text{ }\mu\text{M}$) was two orders of magnitude greater than that of InsP_4 . The inositol trisphosphates tested had a very low affinity [$\text{Ins}(1,3,4)\text{P}_3$, IC_{50} $0.4\text{ }\mu\text{M}$; $\text{Ins}(1,4,5)\text{P}_3$, IC_{50} $6\text{ }\mu\text{M}$; $\text{Ins}(2,4,5)\text{P}_3$, $\text{IC}_{50} > 6\text{ }\mu\text{M}$ [28]]. Completion of our previous analysis of specificity showed that all the newly tested inositol phosphates (see list in legend to Fig. 2b) suppressed InsP_4 binding in cerebellar membranes with affinities at least 500 times lower than that of $\text{Ins}(1,3,4,5)\text{P}_4$. Thus the high specificity of the $\text{Ins}(1,3,4,5)\text{P}_4$ receptor in the membranes *in situ*, which we have described previously [28], was confirmed here. The specificity which we find with the pig cerebellar membranes agrees with that reported by Challiss *et al.* [32] for rat cerebellar membranes.

In comparison with the membranes, the purified receptor displayed a slightly decreased affinity for InsP_4 . This decrease could be due to removal of the natural lipid environment of the receptor protein. Furthermore, as noted previously [17], after receptor purification the pentakisphosphate $\text{Ins}(1,3,4,5,6)\text{P}_5$ inhibited $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ binding with a concentration-dependence similar to that of InsP_4 . It is conceivable that the discrimination between InsP_4 and $\text{Ins}(1,3,4,5,6)\text{P}_5$ might be lost during purification by removing a protein component responsible for this function. Also, Theibert *et al.* [19] report that specificity and affinity of $[^3\text{H}]\text{InsP}_4$ for the solubilized preparation differ from those in the membrane preparation. These authors have provided only very few data on the selectivity of their purified preparation [19,33], which makes a comparison with the results presented here difficult. Moreover it is still unclear whether Theibert *et al.* have isolated a single species or several forms of high-affinity InsP_4 receptors.

In our study the inhibitory potency of all the inositol phosphates tested [apart from $\text{Ins}(1,3,4,5,6)\text{P}_5$] on the purified receptor varied only within a narrow concentration range (Fig. 2a). $\text{Ins}(1,4,5)\text{P}_3$, the only inositol phosphate whose physiological function is clearly shown, even at $10\text{ }\mu\text{M}$ concentration did not affect binding of InsP_4 to the purified receptor.

A major aim of the work presented here was identification of the high-affinity InsP_4 receptor. Previously we have reported the purification of the InsP_4 receptor [18] by chromatography using a cation exchanger (CM-cellulose), an affinity column (heparin-agarose) and a hydroxyapatite column. The maximal binding capacity (B_{max}) was 1500 pmol/mg of protein for the fraction eluted from the hydroxyapatite column and 4600 pmol/mg of protein for the active fraction (0.8 M-NaCl) from a subsequently used second heparin-agarose column [18]. Thus, compared with the original membrane preparation (0.22 pmol of binding sites/mg of protein [28]), we had reached finally an overall purification factor of 20000. Analysis by SDS/PAGE of the fraction with InsP_4 -binding activity obtained after hydroxyapatite chromatography revealed that, besides the 42 kDa protein band, the most likely candidate suggested previously for the high-affinity InsP_4 receptor, there were still several less dominant protein bands (cf. Fig. 2, lane H, in [18]).

Photoaffinity labelling of various fractions obtained during receptor purification (Fig. 3) showed that the label could be displaced by high concentrations of non-radioactive InsP_4 from only one protein band of 42 kDa molecular mass. Thus we have proved here our previous assumption [18] that the protein band of 42 kDa represents the purified high-affinity InsP_4 receptor.

Moreover, the unspecific binding was attributed predominantly to protein bands of 32 and 46 kDa . The possible function of such low-affinity InsP_4 -binding sites still has to be determined. Under the experimental conditions used (concentration of binding sites exceeding that of radioactive ligand) the concentration of free ligand was too low to detect unspecific low-affinity binding in the absence of unlabelled InsP_4 . In addition, it is conceivable that the low-affinity InsP_4 binding site has a higher specificity for AsaInsP_4 than for InsP_4 . Thus $^{125}\text{I-AsaInsP}_4$ available after displacement from the high-affinity binding site (42 kDa protein) is mainly bound to the low-affinity sites (32 kDa protein band and the front of the gel; Fig. 3b) and is hardly displaced by InsP_4 .

In our analysis we could find only the 42 kDa protein band representing a high-affinity specific InsP_4 receptor. No further proteins of higher molecular mass displayed this property, as suggested by the report by Theibert *et al.* [19]. We identified only the 42 kDa protein as a specific InsP_4 receptor protein, whether or not the membrane preparation and the receptor isolation were carried out in the presence of the various proteinase inhibitors listed in the Materials and methods section. But even when proteolysis should still have been occurring, the protein band of 42 kDa described here incorporates the binding domain of the receptor. The purified high-affinity InsP_4 receptor which has been identified here by photoaffinity labelling should allow us to localize this receptor in cells and to determine the physiological functions of InsP_4 in cellular Ca^{2+} homeostasis.

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