cDNA cloning of porcine p42^{IP4}, a membrane-associated and cytosolic 42 kDa inositol(1,3,4,5)tetrakisphosphate receptor from pig brain with similarly high affinity for phosphatidylinositol (3,4,5)P_3

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Abstract **We previously identified a 42 kDa Ins(1,3,4,5)P_4 ($InsP_4$) receptor protein (p42^{IP4}) in brain membranes from several species. Here the cDNA sequence of p42^{IP4} was obtained by PCR using degenerate primers derived from peptide sequences of proteolytic fragments of the porcine protein and by subsequent screening of a pig brain cDNA library. The derived peptide sequence of 374 amino acids for porcine p42^{IP4} is 45 amino acids shorter at the C-terminus than centaurin-α from rat (84% homology) and has a calculated molecular mass of 43 kDa. From the $InsP_4$ binding activity present in brain tissue homogenate about 25% is found in the cytosolic fraction and 75% associated with microsomes. Both activities are due to p42^{IP4} since (i) a peptide-specific antiserum recognizing specifically p42^{IP4} labels the $InsP_4$ receptor protein in membranes and in the cytosol, (ii) the antiserum immunoprecipitates both the membrane protein and the cytosolic protein of 42 kDa, (iii) the $InsP_4$ binding activity released by high salt or by alkaline extraction from membranes is identified immunologically as the 42 kDa protein, and (iv) the affinity for $InsP_4$ and specificity for various inositolphosphates are similar for the membrane-associated and for the soluble p42^{IP4}. The functional importance of p42^{IP4} is highlighted by the identical affinity for $InsP_4$ and for phosphatidylinositol (3,4,5)P_3 (K_i = 1.6 and 0.9 nM, respectively). Thus, the $InsP_4$ receptor, apparently a peripheral membrane protein, which exists also as a cytosolic protein can transfer the signals mediated by $InsP_4$ or by $PtdInsP_3$ between membranes and cytosolic compartment.**

Key words: Signal transduction; Inositol phosphate; Phosphatidylinositol 3-kinase; Ins-P_4; $PtdInsP_3$; Centaurin-α

1. Introduction

We do not yet have clear evidence about the role of the putative second-messenger inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) a metabolite of the well-characterized second-messenger inositol 1,4,5-trisphosphate ($InsP_3$) which releases Ca^{2+} from intracellular stores [1,2]. Some physiological functions of $InsP_4$ proposed so far are connected to cellular Ca^{2+} control. The diverse effects attributed to $InsP_4$, such as re-sequestration of Ca^{2+} into the internal stores [3], activation of Ca^{2+} release from stores [4], influx of Ca^{2+} into non-excitable cells [5] or involvement in generation of cytosolic Ca^{2+} oscillations [6] reflect conflicting evidence for the site of action of $InsP_4$. Thus, $InsP_4$ most likely acts via several different receptor proteins. This is consistent with reports about different $InsP_4$ binding proteins in neural and in non-neural tissue. In pig platelets a 104 kDa $InsP_4$ receptor protein has been identified as a member of the GAPl family [7]. In rat brain, oligomeric complexes binding $InsP_4$ and/or $InsP_6$ have been described [8].

Previously in our laboratory a receptor protein with high affinity for Ins(1,3,4,5)P_4 was solubilized from pig cerebellar membranes and purified [9]. The protein was identified in SDS-PAGE after photoaffinity labelling as a 42 kDa protein band, p42^{IP4} [10]. Peptide sequences obtained from this protein after proteolytic digestion enabled us to develop peptide-specific antisera [11,12]. One of these antisera directed against peptide-3 (19 amino acids) recognized the intact receptor protein in ELISA and in Western blot and immunoprecipitated the detergent-solubilized $InsP_4$ receptor protein [12]. In the present study we report cloning of the cDNA for p42^{IP4} from pig brain. The cDNA sequence showed a high homology to the recently cloned centaurin-α from rat brain [13] and to several human EST clones. Moreover, we demonstrate that a 42 kDa protein in the cytosolic fraction recognized by the antiserum is a soluble form of the $InsP_4$ receptor protein.

For the putative messenger molecule PtdIns(3,4,5)P_3 no high-affinity receptor protein has been found so far. Several studies had shown that inositol phosphates and phosphoinositide lipids with identical head groups did not bind with the same affinity to the same binding site; examples are the comparison of Ins(1,4,5)P_3 with PtdIns(4,5)P_2 [14] or Ins(1,3,4,5)P_4 with PtdIns(3,4,5)P_3 [15,16]. Nevertheless, here we see that p42^{IP4} has identical affinities for both ligands. A homologous protein from rat brain of different molecular mass, centaurin-α [13], was described as a $PtdInsP_3$ binding protein. Moreover, we report that the membrane-associated p42^{IP4} and the cytosolic form have the same high affinity for $PtdInsP_3$ and for $InsP_4$. Thus, both signaling molecules, $InsP_4$ and PtdIns(3,4,5)P_3, might converge in their action on the 42 kDa protein p42^{IP4}.

2. Materials and methods

2.1. Binding assays

Membranes were prepared from pig cerebellum in the presence of protease inhibitors (0.2 mM Pefabloc SC, 1 mM EGTA, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM benzamidin) as described previously [12], with the modification to perform all centrifugation steps at 100 000 × *g*. The binding assays with fractions obtained during membrane preparation were performed in a final volume of 400 μl in

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Abbreviations: CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; GroPIP$_3$, glycero phosphatidylinositol(3,4,5) trisphosphate; PtdIns(3,4,5)P_3 ($PtdInsP_3$), phosphatidylinositol(3,4,5)-trisphosphate; Ins(x_1, x_2,..,x_n)P_n, myo-inositol(x_1 ,x_2,..,x_n)n-phosphate

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microcentrifuge tubes. The samples (usually 0.2 mg of protein per assay) were incubated at 4°C for measurement of (i) $InsP_4$ binding activity in a solution containing 0.9–1.2 nM D-[^{3}H]Ins(1,3,4,5)P_4 (0.77 TBq/mmol, NEN DuPont, Bad Homburg, Germany; ≈20 000 dpm), 0.25% BSA, 1 mM EDTA, 25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, and (ii) of $InsP_3$ binding activity in the presence of 0.5–0.6 nM D-[^{3}H]Ins(1,4,5)P_3 (1.59 TBq/mmol, Amersham, Braunschweig, Germany; ≈20 000 dpm), 0.1% BSA, 1 mM EDTA, and 25 mM TRIS-HCl, pH 9.0. For $InsP_4$ binding in some experiments, as described in [17] buffers with pH 5.5 and 7.0 or the buffer with pH 7.4 employed by Theibert and coworkers [13] were used. Nonspecific binding was determined in the presence of 1 μM D-Ins(1,3,4,5)P_4 (Cell Signal Inc., Kingston, RI) or D-Ins(1,4,5)P_3 (BIOMOL, Hamburg, Germany), respectively. The samples were incubated in the presence of different concentrations of unlabelled inositolphosphates for 20 min. Bound ligand was separated from free ligand by adding 100 μl globulin (10 mg/ml) and 500 μl 5% PEG-8000, further incubating for 15 min at 4°C and then centrifuging (14 000×*g*, 15 min, 4°C). The pellets were washed with 100 μl of binding buffer and then solubilized with 2% SDS before transferring into scintillation vials and measuring the bound radioactivity. All determinations made in duplicate or triplicate showed less than 15% deviations and were repeated at least twice. For the analysis of the data the RADLIG computer program (Version 4; BIOSOFT, Cambridge, UK) was used to estimate K_d values and the number of binding sites as described [17,18].

2.2. Extraction of the $InsP_4$ receptor from membranes

Aliquots of the membranes prepared from pig cerebellum (5 mg of each protein) were centrifuged and the pellets were resuspended in 1 ml of (i) homogenization buffer [12], (ii) 100 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), pH 11.0, (iii) homogenization buffer supplemented with 1 M NaCl, or (iv) 1% (v/v) mercaptoethanol in homogenization buffer and incubated for 1 h on ice. The samples were then centrifuged (100 000×*g*, 30 min, 4°C) and the supernatants analyzed by Western blotting with the antipeptide antiserum. SDS-PAGE and immunoblotting were carried out as described [12]. Immunoprecipitation of the $InsP_4$ receptor protein was made as published previously [12] using aliquots of the cytosolic fraction (1 mg of protein).

2.3. Protein sequencing and cDNA cloning

Partial peptide sequences were obtained from protein fragments after treating $p42^{IP4}$ with CNBr [11] (sequences designated CB-n, underlined in Fig. 1) or with LysC [12] (sequences designated K-m, underlined in Fig. 1). The peptide sequences CB-1, CB-4, CB-5, K-9 and K-14 were used to design sense and antisense degenerate oligonucleotide primers. These oligonucleotides were employed for PCR with cDNA synthesized by reverse transcription of total RNA isolated from pig cerebellum primed with oligo(dT). The PCR conditions were 5 min at 94°C for denaturation, and 35 cycles at 94°C for 1 min, at 50°C for 1.10 min, at 72°C for 1 min for amplification and 5 min for final elongation at 74°C. The amplified products were separated on a 7% polyacrylamide gel; bands were cut out, eluted and cloned into the pCR-Script cloning vector (Stratagene, Heidelberg, Germany). After transformation of XL1-blue *Escherichia coli* cells, clones with insert were sequenced. PCR clones with sequences corresponding to peptide sequences were random labelled with [^{32}P]dCTP (Amersham) and used to screen a λ gt11 porcine cortex cDNA library (Clontech, Heidelberg, Germany). Positive phage plaques were rescreened twice and five independent single plaques were picked. DNA was isolated according to the protocol given by the manufacturer. After size determination of inserts by restriction digestion inserts were subcloned into pUC-derivative cloning vector for sequencing. The $p42^{IP4}$ cDNA sequence from pig brain was confirmed by sequencing two independent overlapping clones on both strands using the dideoxy chain termination method. Sequence analysis was performed with the software package of the University of Wisconsin Genetics Computer Group.

3. Results and discussion

Previously in our laboratory we purified and characterized the 42 kDa inositol(1,3,4,5) tetrakisphosphate receptor from pig cerebellum, $p42^{IP4}$ [9,10]. The protein was digested with LysC or with BrCN [11,12]. Degenerate oligonucleotide primers derived from these peptides were used to generate by RT-PCR clones using oligo(dT) primed cDNA from pig brain RNA (clones 1, 2, 3 corresponding to nucleotides 363–736; 648–1003 and 945–1210 in Fig. 1). The resulting cDNA was employed as a probe to screen a pig brain cDNA library. The complete nucleotide sequence for $p42^{IP4}$ containing the entire coding region of 1122 bp, sequenced in both directions is represented in Fig. 1 with the resulting peptide sequence. The encoded protein of 374 amino acids contains all the sequenced cleavage products and has a calculated molecular mass of 43 kDa, very close to the experimental value of 42 kDa. The excess of basic amino acid residues is consistent with the chromatographic behaviour of this protein (binding to cation exchanger at pH 7.0).

The protein sequence of $p42^{IP4}$ from pig brain is 84% homologous to that reported for centaurin-α, a PtdIns(3,4,5)P_3 binding protein from rat brain [13], whereas $p42^{IP4}$ is 45 amino acid residues shorter at the C-terminus than centaurin-α. $p42^{IP4}$ shows no distinct hydrophobic domain corresponding

Table 1
Pharmacology of D-[^{3}H]Ins(1,3,4,5)P_4 binding to membranes and cytosolic fraction from pig cerebellum

$InsP_n$	Membranes ($K_i \pm \sigma_n$)	Cytosol ($K_i \pm \sigma_n$)
D-Ins(1,3,4,5)P_4	1.65 ± 0.46 nM	3.82 ± 0.84 nM
D/L-Ins(1,3,4,5)P_4 = L/D-Ins(1,3,5,6)P_4	8.86 ± 4.99 nM	5.78 ± 1.52 nM
D/L-Ins(3,4,5,6)P_4 = L/D-Ins(1,4,5,6)P_4	150.8 ± 56.5 nM	261.0 ± 95.6 nM
Ins(1,3,4,6)P_4	217 ± 30.9 nM	166.2 ± 6.5 nM
D-GroPIP$_3$	0.91 ± 0.11 nM	2.74 ± – nM
D/L-Ins(1,4,5)P_3 = L/D-Ins(3,5,6)P_3	354.3 ± 109.5 nM	194.3 ± 19.6 nM
D-Ins(1,4,5)P_3 = L-Ins(3,5,6)P_3	3.52 ± 0.07 μM	5.7 ± 2.2 μM
D/L-Ins(1,5,6)P_3 = L/D-Ins(3,4,5)P_3	17.1 ± 6.9 nM	31.2 ± 1.7 nM
D-Ins(1,2,6)P_3 = L-Ins(2,3,4)P_3	3.31 ± 1.43 μM	6.1 ± 1.16 μM
D/L-Ins(1,2,4,5,6)P_5 = L/D-Ins(2,3,4,5,6)P_5	341.0 ± 4.3 nM	564.2 ± 147.8 nM
Ins(1,3,4,5,6)P_5	37.2 ± 13.8 nM	84.7 ± 11.4 nM
D/L-Ins(1,2,3,4,5)P_5 = L/D-Ins(1,2,3,5,6)P_5	18.0 ± 10.9 nM	14.2 ± 1.8 nM

The efficacy of D-GroPIP$_3$ and of various InsP-isomers to displace D-[^{3}H]Ins(1,3,4,5)P_4 was tested using the membranes and the cytosolic fraction from pig cerebellum in the presence of 1.1 nM (in the case of D-GroPIP$_3$ 1.3 nM) D-[^{3}H]Ins(1,3,4,5)P_4 in buffer pH 5.0. The small amount of GroPIP$_3$ available allowed determination of displacement of D-[^{3}H]Ins(1,3,4,5)P_4 by this compound in the cytosolic fraction in one experiment only. Nonspecific binding was determined in all cases at 1 μM D-Ins(1,3,4,5)P_4. In each experiment the K_i value was obtained from a displacement curve testing the compound at 4–8 different concentrations. For D-Ins(1,3,4,5)P_4 the mean value was derived from 9 (6) experiments for membranes (cytosol), for the other compounds from 2 to 3 experiments.

to a putative membrane spanning region. From the 10 ankyrin-like repeats in the rat protein we find eight in $p42^{IP4}$. Moreover, $p42^{IP4}$ contains the putative $InsP_n$ binding protein consensus sequence [13] and most of the consensus amino acids in ankyrin repeat six (amino acids 259–302), which are homologous to proteins of the TERM family (talin, ezrin, radixin, moesin and merlin). Also the hypothesized zinc coordination site in centaurin-α was detected in $p42^{IP4}$ from pig. The functional significance of these structural motifs has to be clarified.

We further investigated whether $p42^{IP4}$ occurs not only in membranes but also in the cytosol. Using an antipeptide antiserum produced against a 19 amino acid peptide (peptide-3 [12] corresponding to K-14 in Fig. 1) derived from peptide sequencing of fragments of porcine $p42^{IP4}$ we were able to detect this protein in the membrane fraction [12]. This tool was employed to elucidate whether there is a soluble form of the Ins(1,3,4,5)P_4 receptor in cerebellum. Therefore we firstly made a comparative analysis of binding of D-[^{3}H]Ins(1,3,4,5)P_4 and D-[^{3}H]Ins(1,4,5)P_3 in the fractions of the membrane preparation and, secondly, we examined the same fractions by Western blot (Fig. 2). To rule out the possibility that the results obtained with a soluble fraction were partly due to contaminating membranes the formerly used preparation protocol (sedimenting at $35\,000 \times g$) was modified by carrying out all centrifugation steps at $100\,000 \times g$.

From the D-[^{3}H]Ins(1,3,4,5)P_4 binding activity found in the tissue homogenate about 25% appeared in the supernatant from the centrifugation ($100\,000 \times g$) whereas the other 75% were located in the microsomal fraction (Fig. 2Aa-c). Washing the membranes with buffer supplemented with 400 mM NaCl caused the release of a small amount of Ins(1,3,4,5)P_4 binding activity from the membranes (Fig. 2Ad) whereas in the following low salt washing fractions no D-[^{3}H]Ins(1,3,4,5)P_4 binding was detectable. On the other hand, D-[^{3}H]Ins(1,4,5)P_3 binding activity was found in the membrane fraction (Fig. 2Ac,e,g) but not in the cytosolic fraction (Fig. 2Ab). Some D-[^{3}H]Ins(1,4,5)P_3 binding activity was detected in the high salt wash fraction (Fig. 2Ad). It should be noted that the recovery of D-[^{3}H]Ins(1,3,4,5)P_4 binding activity was almost 100%, whereas approximately 50% of the initially determined D-[^{3}H]Ins(1,4,5)P_3 binding activity were lost during membrane preparation, possibly by inactivation of the binding proteins.

Fig. 2B shows the corresponding Western blot developed by using the antipeptide antiserum as described earlier [12]. The 42 kDa $InsP_4$ receptor protein was the prominent protein band detected in the homogenate (lane 1), the cytosolic fraction (lane 2), the membranes prepared from pig cerebellum (lane 3), which can be compared with purified $InsP_4$ receptor in lane 4. A protein of 25 kDa which was labelled in the homogenate (lane 1) was enriched in the cytosolic (lane 2) and the washing fractions but only a minor constituent of the membranes (lane 3). Another membrane-associated protein was recognized at 37 kDa by the antipeptide antiserum in some preparations as demonstrated previously [12]. Interestingly, only in the cytosol (lane 2) a protein in the high molecular mass range (>100 kDa) was labelled with intensities varying between different preparations. The nature of this protein still has to be clarified.

The 42 kDa $InsP_4$ binding protein appears to some degree in the fraction obtained by washing the membranes with buffer containing 400 mM NaCl. To investigate the characteristics of the interaction of $p42^{IP4}$ with the membrane, membranes were incubated with (i) homogenization buffer (pH 7.7, control), (ii) 100 mM CAPS, pH 11.0, and homogenization buffer containing (iii) 1 M NaCl or (iv) 1% mercaptoethanol, and centrifuged. The supernatants were analyzed by Western blotting with the antipeptide antiserum (Fig. 3). The results show that the 42 kDa protein is solubilized from the membranes at the basic pH (lane 3) and by the high salt concentration (lane 4) but neither by treatment with the homogenization buffer (lane 2) nor by the presence of 1% mercaptoethanol (lane 5). This indicates that the 42 kDa protein is not an integral membrane protein but a peripheral membrane protein which is associated with the membrane by ionic interactions and probably not anchored through covalent modifications like attachement of lipids. Interestingly the 25 kDa protein which was also recognized by the antipeptide antiserum was enriched in the pH 11 supernatant (lane 3) but did not appear in the other supernatants.

The results obtained with Western blotting and analysis of D-[^{3}H]Ins(1,3,4,5)P_4 binding showed that the 42 kDa $InsP_4$ receptor exists both in a membrane-associated and in a soluble form. To further underpin this conclusion, we tested whether it was possible to immunoprecipitate the soluble form of the receptor from the cytosolic fraction with the antipeptide antiserum. The Western blot analysis of such an experiment (Fig. 4) shows that the 42 kDa $InsP_4$-receptor protein was immunoprecipitated from the cytosolic fraction by the antiserum (lane 2). The 25 kDa protein which was also recognized in the cytosolic fraction (lane 1) was not immunoprecipitated. Previously we have reported that the antipeptide antiserum could immunoprecipitate the Ins(1,3,4,5)P_4 receptor solubilized by detergent treatment of porcine cerebellar membranes and enriched by a following CM-cellulose chromatography [12]. Similarly to the experiments using cytosol, the 25 kDa protein is not immunoprecipitated from detergent-solubilized membranes (not shown). The precipitation of the cytosolic 42 kDa $InsP_4$-receptor protein was specifically inhibited by preincubation of the antibodies with the peptide used to generate the antiserum (Fig. 4, lane 3). No protein was precipitated by the protein A-sepharose in the absence of the antiserum (lane 4).

Within the cytosol there are different proteins which can bind inositolphosphates. A typical example is the report by Kanematsu et al. [19] of the purification of two proteins from the cytosolic fraction of rat brain which bind Ins(1,4,5)P_3 with high affinity (K_d of 2–5 nM). One of these proteins with a molecular mass of 85 kDa was identified as the δ1-isozyme of the phospholipase C [19] and the other as a 130 kDa Ins(1,4,5)P_3 binding protein homologous to phospholipase Cδ-1 [14].

For characterizing the Ins(1,3,4,5)P_4 binding site of the cytosolic fraction we determined the affinity for Ins(1,3,4,5)P_4 and for other inositolphosphates and compared these results with the data obtained for the membrane fraction. Scatchard analysis of the displacement curves for D-Ins(1,3,4,5)P_4 gave a K_d value of 1.65 nM for the microsomal fraction (B_{max}: 286 fmol/mg; $n=9$) and a K_d value of 3.8 nM for the cytosolic fraction (B_{max}: 386 fmol/mg; $n=6$). In both cases a two binding site model can be excluded because it does not fit the data significantly better than a one binding site model. Heterologous displacement of D-[^{3}H]Ins(l,3,4,5)P_4 by D-Ins(1,4,5)P_3, D-Ins(1,2,6)P_3 and Ins(1,3,4,5,6)P_5 showed nearly identical displacement curves for the soluble and the membrane-associated

Fig. 1. Nucleotide sequence and deduced amino acid sequence of cloned cDNA of pig $p42^{IP4}$. (A) The numbers of nucleotides are indicated along the right margin above the number of the amino acid residue. Underlining of amino acid sequences shows the peptide sequences obtained by microsequencing of CNBr fragments (CB-1, CB-4, CB-5) or LysC fragments (K-2, K-4, K-6, K-9, K-11, K-14). CB-5 was further confirmed by proteolytic fragments K-7 and K-12. (B) Amino acid sequence alignment of pig $p42^{IP4}$ and rat centaurin-α. Gen bank accession number U88368.

Ins(1,3,4,5)P_4 receptor (analysis summarized in Table 1). D-Ins(1,2,6)P_3, α-trinositol, is an interesting isomer because it is of potential therapeutic use as an anti-inflammatory compound [20]. Previously we characterized the binding sites for α-trinositol in various tissues and compared those with the Ins(1,3,4,5)P_4 binding sites [17]. Most importantly, the water-soluble phosphatidyl-inositol(3,4,5) trisphosphate-derivative D-GroPIP$_3$ displaced D-[^{3}H]Ins(1,3,4,5)P_4 from binding to the membranes and the cytosolic fraction with an affinity equal to or even slightly higher than that of D-Ins(1,3,4,5)P_4 itself.

We determined binding of [^{3}H]InsP_4 to $p42^{IP4}$, either membrane attached or detergent solubilized at various binding conditions (Fig. 3B): binding in (i) acetate/phosphate buffer at pH 5.0 (see Section 2), or in a buffer containing 20 mM NaCl and 100 mM KCl at (ii) pH 7.0 or (iii) pH 5.5 (as used in [21], details are published in [17]) is compared with binding (iv) at pH 7.4 with low salt concentration (25 mM Tris-HCl, 1 mM EDTA, 0.5 mM phosphate and 0.5 mM pyrophosphate) as in [13]. We detected the highest InsP_4 binding activity in the pH 5.0 buffer, whereas both buffer (ii) and (iii) yielded a 50% reduced amount of specific binding at a tracer concentration of 1.2 nM. At pH 7.0 the K_d was 42 ± 8 nM ($n = 3$) and B_{max} was 3.2 ± 0.5 pmol/mg protein. Thus, the affinity was reduced slightly, 20-fold by the pH change from 5.0 to 7.0. At the low salt conditions, pH 7.4, no InsP_4 binding could be detected. This is in agreement with the data reported by Theibert's group, which could not detect binding of [^{3}H]InsP_4 to centaurin-α under these conditions [13,24]. Obviously photoaffinity labeling with a lipophilic ligand is still possible under these conditions. Interestingly another InsP_4 binding protein, the $GAPI^{IP4}$ investigated by Cullen et al. from pig platelets displays maximal binding activity at pH 5.5–6.0 (Fig. 1 in [21]). Moreover, InsP_4 binding activity was maximal at pH 5.0 in canine smooth muscle membranes [22], as in cerebellar membranes [23]

Previously, using the same experimental conditions described here we systematically characterized the binding site of the purified InsP_4 receptor by testing all inositol tris-, tetrakis-, and pentakisphosphate regioisomers [18]. Some of the inositolphosphates which are active at the purified receptor protein were selected for the present comparative study of the characteristics of the cytosolic and the membrane-bound receptor. The analysis in Table 1 revealed that there was no significant difference in the K_i values obtained either with membranes or with cytosol. The racemic mixture D/L-

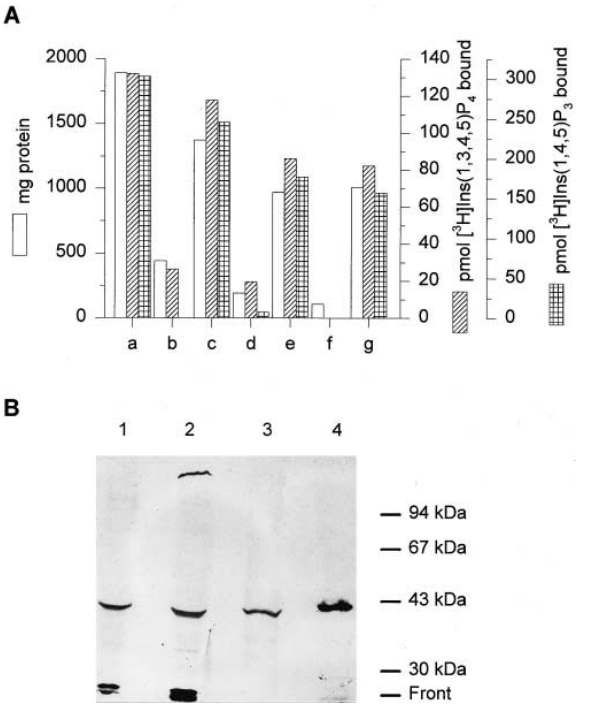

Fig. 2. Analysis of the fractions obtained during preparation of membranes from pig cerebellum: (A) binding of D-[^{3}H]Ins(1,3,4,5)P_4 and D-[^{3}H]Ins(1,4,5)P_3 and (B) distribution of the Ins(1,3,4,5)P_4-receptor protein by Western blot. The homogenate from pig cerebellum (Aa; B, lane 1) was centrifuged giving the cytosolic fraction (Ab; B, lane 2) and the pellet (Ac). After removal of the supernatant the pellet was resuspended in homogenization buffer supplemented with 400 mM NaCl and centrifuged again. The resulting supernatant (Ad) was removed and the pellet (Ae) was washed twice by resuspension and centrifugation. The supernatants from these wash steps were pooled (Af), whereas the membrane pellet (Ag; B, lane 3) was resuspended in homogenization buffer. The binding assay was carried out in the presence of 1.2 nM D-[^{3}H]Ins(1,3,4,5)P_4 or 0.58 nM D-[^{3}H]Ins(1,4,5)P_3 and 200 μg protein per assay (in Af 70 μg of protein were used) as described in Section 2. After an incubation for 20 min at 4°C bound ligand which was separated from free ligand was quantified by measuring radioactivity. In lanes 1–3 in (B) 200 μg of protein were separated by SDS-PAGE (10% separation gel). For comparison in lane 4 purified Ins(1,3,4,5)P_4 receptor was applied (≈200 ng of protein). After SDS-PAGE followed by electroblotting to a PVDF-membrane the blot was developed with antipeptide-antiserum.

Ins(1,3,4,5)P_4 displayed 2–6 times lower affinity than the stereochemically pure D-isomer. This indicates that the L-isomer is inactive in displacing D-[^{3}H]Ins(1,3,4,5)P_4 from the membrane-associated and the cytosolic receptor. With Ins(1,4,5)P_3 the opposite effect was seen. The racemic mixture D/L-Ins(1,4,5)P_3 was approximately 10 times more potent than D-Ins(1,4,5)P_3 in both membranes and cytosol (200–350 nM versus 3–6 μM, Table 1). This suggests that L-Ins(1,4,5)P_3 is the displacing agent. It should be noted that L-Ins(1,4,5)P3 is identical to D-Ins(3,5,6)P_3. Another interesting InsP_3-isomer is the racemic mixture D/L-Ins(1,5,6)P_3, which is only by a factor of 10 less potent in inhibiting D-[^{3}H]Ins(1,3,4,5)P_4 binding than the D-Ins(1,3,4,5)P_4 molecule (17.1 and 31.2 nM versus 1.6 and 3.8 nM for the membranes and the cytosolic fraction, respectively; see Table 1). Since L-Ins(1,5,6)P_3 is identical with D-Ins(3,4,5)P_3 we conclude from our data that this compound is the displacing agent but not D-Ins(1,5,6)P_3. The relative order of affinities of the inositoltetrakisphosphate regioisomers which we have studied in detail with solubilized and purified protein [18] is even strikingly similar to the order of affinities reported for a different InsP_4 binding protein GAP^{IP4} [21], notably determined at different binding conditions, at pH 7.0.

Within the InsP_5 isomers tested (two enantiomeric pairs and one mesoform) D/L-Ins(1,2,4,5,6)P_5 was the weakest ligand (K_i = 341 and 564 nM with the membranes and the cytosolic fraction, respectively; Table 1) whereas Ins(1,3,4,5,6)P_5 and D/L-Ins(1,2,3,4,5)P_5 showed only 10–20-fold lower affinities than D-Ins(1,3,4,5)P_4. It is interesting to note that the affinity of the InsP_4 receptor for Ins(1,3,4,5,6)P_5 increased to values which were comparable to the K_d for D-Ins(1,3,4,5)P_4 when the receptor protein was solubilized with detergent from the membranes [9], whereas the K_i of the soluble, cytosolic receptor reported here was as low as that of the membrane-attachted receptor.

Previously, we observed that a modification at the 1-position (photoaffinity analogue) of Ins(1,3,4,5)P_4 reduced the affinity [10]. In this context our present finding is of great importance that the phosphatidylinositol(3,4,5)trisphosphate-derivative D-GroPIP_3 in which the phosphate group at C-1 of the inositol ring is coupled to glycerol displaced D-[^{3}H]Ins(1,3,4,5)P_4 in membranes with an inhibitory constant of 0.9 nM and in the cytosolic fraction with 2.7 nM (Table 1). The same high affinity was seen with the InsP_4 receptor protein purified from membranes [18]. Therefore it is possible that this compound is a physiological ligand for the InsP_4 binding protein p42^{IP4}. A detailed analysis of physiological conditions which control ligand binding and affinity of p42^{IP4} might give us a clue about the cellular function of this protein. Binding of the highly phosphorylated ligands could be affected by salt concentration, in particular, pH (protonation/deprotonation of the inositololigophosphates or of charged amino acid residues in the binding protein) or by additional protein factors possibly interacting in the intact cell.

Theibert et al. [8,24] used an InsP_4 affinity column to isolate specific high-affinity InsP_x binding proteins from rat cerebellar membranes. Using the same strategy this group has recently isolated and cloned another inositolphosphate binding protein of 46 kDa from rat brain which they called centaurin-α [13]. This protein exists in a membrane-associated and in a soluble form. Like p42^{IP4}, purified natural centaurin-α has been shown to bind both PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4. The difference in affinities deduced (IC_{50} values of 120 and 620 nM for PtdInsP_3 and Ins(1,3,4,5)P_4, respectively) is probably due to the different experimental conditions used for the binding assay. Centaurin-α has a clear selectivity for C-1 and C-3 phosphorylated inositolphosphates compared to other inositolphosphates tested. Therefore this protein was proposed to be a high affinity PtdInsP_3 binding protein.

The 42 kDa InsP_4 receptor p42^{IP4} and centaurin-α are closely related InsP_4 and PtdInsP_3 binding proteins, with p42^{IP4} being 45 amino acid residues shortened at the C-terminus (Fig. 1B). Centaurin-α identically contains peptide-3 from the 42 kDa InsP_4 receptor from pig cerebellum which we used previously for the generation of the antipeptide antisera [12]. Using membranes from different parts of the rat brain or retina we found by Western blotting with this antiserum a protein comigrating in SDS-PAGE with the 42 kDa protein from pig cerebellum (data not shown). This antiserum, how-

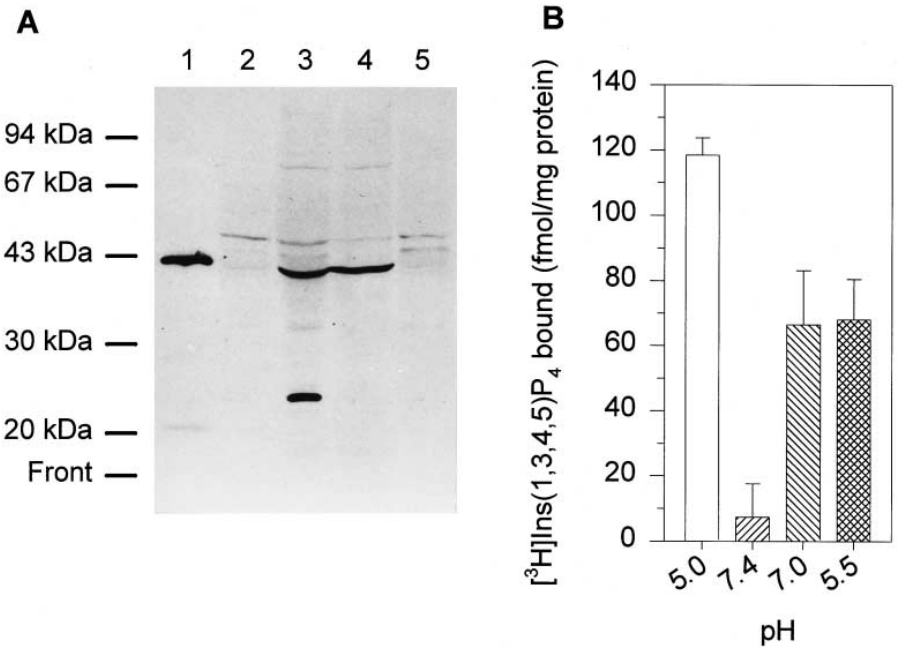

Fig. 3. Characteristics of $p42^{IP4}$. (A) Extraction of the $InsP_4$ receptor from membranes. Aliquots of the membranes were treated as described in Section 2 with homogenization buffer (lane 2), 100 mM CAPS, pH 11.0 (lane 3) or homogenization buffer containing 1 M NaCl (lane 4) or 1% mercaptoethanol (lane 5). 300 μl of the resulting supernatants were applied in each lane. In lane 1, 100 ng purified $InsP_4$ receptor protein was loaded on the gel. After SDS-PAGE (12.5% separation gel) the proteins were blotted to a PVDF membrane. The blot was developed with the antipeptide antiserum [12]. (B) Influence of pH on $InsP_4$ binding of $p42^{IP4}$. Specific binding of [^{3}H]Ins(1,3,4,5)P_4 (1.2 nM) to membranes (200 μg/assay). Buffers with pH 5.0 are described in [23], pH 5.5 and 7.0 in [21,17], pH 7.4 in [13,24]. Data presented are mean ± SD from two separate experiments measured in triplicate.

ever, did not detect a protein from rat brain at 46 kDa (cf. Fig. 4 in [12]).

PtdIns 3-kinase which catalyzes the phosphorylation of inositol phosphate lipids at the 3-position of the inositol ring

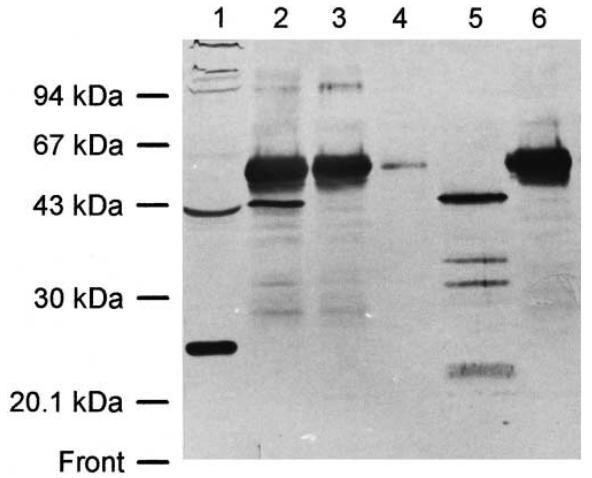

Fig. 4. Immunoprecipitation of the Ins(1,3,4,5)P_4 receptor protein from the cytosolic fraction analyzed by SDS-PAGE and Western blot using antipeptide antiserum. Samples of the cytosolic fraction (lane 1) containing 1 mg of protein each were used for the experiments as described in Section 2 for the precipitation assay (lane 2), competition by peptide-3 (lane 3) and the control experiment (lane 4), respectively. For comparison the purified $InsP_4$ receptor from pig cerebellar membranes (lane 5, 100 ng of protein) and antipeptide antiserum (lane 6, 3 μg of protein) were also subjected to SDS-PAGE and Western blot. In lane 5 the protein bands detected in the molecular mass range below 42 kDa result from partial degradation of the purified receptor protein.

[25] is activated by a range of diverse cell surface receptors such as G-protein-coupled receptors, growth factor and cytokine receptors [26]. PtdIns 3-kinase is a heterodimer of a 85 kDa adaptor subunit (p85) and a 110 kDa catalytic subunit (p110) ([16] and refs. therein). The p85 regulatory subunit has several domains exhibiting characteristic homology to proteins involved in signal transduction cascades: a Src homology (SH) 3 domain, two SH 2 domains and a region homologous to the GTPase-activating proteins (GAP) characteristic for the small GTP binding proteins [26]. In vivo, activation of PtdIns 3-kinase by various receptors leads to the rapid appearance of PtdIns(3,4,5)P_3 in cells and this lipid has been postulated to act as a second messenger conveying information from the receptor to yet unidentified intracelluar targets [26]. PtdIns 3-kinase can be inhibited specifically by Wortmannin. This tool has allowed to identify an important role for this enzyme and its metabolite in various cell functions (for review see [27]): these include activation of glucose transport and nerve growth factor stimulated neurite elongation. In endothelial cells PDGF receptor stimulation causes rearrangement of actin filaments resulting in membrane ruffling through activation of PtdIns 3-kinase [28]. The signal is mediated by activation of the small G-protein Rac by PtdIns(3,4,5)P_3 [29], thus inducing a selective subset of cellular responses [30].

So far there is hardly any evidence about the nature of the target proteins of PtdIns(3,4,5)P_3. The only striking example known so far is that this lipid specifically interacts with the SH2 domain of PtdIns 3-kinase thus inhibiting the association of the kinase with tyrosine-phosphorylated proteins [16]. Since the 42 kDa protein might be a specific receptor for PtdIns(3,4,5)P_3 this protein $p42^{IP4}$ and Centaurin-α could play a crucial role in PtdInsP_3-kinase-dependent signal trans-

duction. Moreover, the comparably high affinity of the 42 kDa protein for PtdIns(3,4,5)P_3 and for Ins(1,3,4,5)P_4 makes it possible that this protein mediates a link between the inositol polyphosphate and the phosphatidylinositol polyphosphate signal pathways. The high abundance of the 42 kDa protein in well-defined brain structures (Kreutz and Reiser, unpublished) suggests that $p42^{IP4}$ is involved in neuron-specific signalling events. An $InsP_4$ recognition site was found on synaptotagmin, a protein involved in Ca^{2+} dependent vesicle fusion [31]. Furthermore a block by $InsP_4$ of the assembly of the docking particle for synaptic release has been reported [32]. Thus, $InsP_4$ might play a role in neuronal plasticity modulating transmitter release.

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