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Determination of Specificity of a High-Affinity Inositol 1,3,4,5-Tetrakisphosphate Binding Site at a 42 kDa Receptor Protein, $p42^{IP4}$: Comparison of Affinities of All Inositoltris-, -Tetrakis-, and -Pentakisphosphate Regioisomers

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The specificity of the binding site of $p42^{IP4}$, a high-affinity 42 kDa Ins $(1,3,4,5)P_4$ receptor protein identified by photoaffinity labelling (Reiser *et al., Biochem. J.* **280,** 533, 1991) was analyzed by determining the affinities for all possible inositoltris-, -tetrakis-, and -pentakisphosphate regioisomers. We tested the purified receptor protein displaying a K_d of 2.2 nM for Ins $(1,3,4,5)P_4$ which was unequalled by any of the other inositoltetrakis- and -trisphosphate regioisomers. The affinities of inositoltetrakisphosphates were 25 to 150 times lower, with a substitution at C-2 having the largest effect in reducing the affinity. The inositoltrisphosphate isomers were three orders of magnitude less potent than Ins $(1,3,4,5)P_4$, apart from D/L-Ins $(3,4,5)P_3$. The pentakisphosphate Ins$(1,3,4,5,6)P_5$ had an affinity for the solubilized and purified receptor comparable to that of D-Ins$(1,3,4,5)P_4$. This lack of discrimination was unique for the solubilized receptor, since it was not observed with the membrane-associated receptor protein. Most importantly, D-Ins$(1,3,4,5)P_4$ and D-Gro PtdIns$(3,4,5)P_3$ had identical affinities with the 42 kDa protein. Thus, this protein $p42^{IP4}$ selectively recognizes two potential second messengers.

D-myo-inositol 1,4,5-trisphosphate (D-Ins$(1,4,5)P_3$), a product of receptor -activated hydrolysis of phosphatidylinositol 4,5-bisphosphate, is a well established second messenger in Ca^{2+}-signalling by releasing Ca^{2+} from intracellular stores via Ins$(1,4,5)P_3$-gated channels [1, 2]. D-Ins$(1,4,5)P_3$ is metabolized partially by dephosphorylation catalysed by $InsP_3$ 5-phosphatase, yielding D-Ins$(1,4)P_2$ which is further metabolized to free inositol. Alternatively, phosphorylation leads to D-inositol 1,3,4,5-tetrakisphosphate (D-Ins$(1,3,4,5)P_4$) through the action of a $InsP_3$ 3-kinase (reviewed in [3]).

D-Ins$(1,3,4,5)P_4$ has been suggested to be a second messenger regulating the intracellular Ca^{2+} concentration but its precise function is still largely unknown. Specific high-affinity binding sites for D-Ins$(1,3,4,5)P_4$ have been found in a wide range of tissues [4]. The subcellular distribution of Ins$(1,3,4,5)P_4$ binding sites has been studied in human platelets [5] and rat liver [6].

Ins$(1,3,4,5)P_4$ binding proteins have been purified from different species and tissues. A 104 kDa $InsP_4$ receptor purified from pig platelets [7] identified by cDNA sequencing codes for a member of the GTPase-activating protein (GAP) family and was therefore named $GAP1^{IP4BP}$ [8]. Another 104 kDa $InsP_4$ binding protein probably identical to $GAP1^{IP4BP}$ was recently

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Abbreviations: CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; $GroPIP_3$, glycerophosphatidylinositol(3,4,5) trisphosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PtdIns$(3,4,5)P_3$, phosphatidylinositol(3,4,5) trisphosphate; Ins$(x_1, x_2, \ldots, x_n)P_n$, myo-inositol$(x_1, x_2, \ldots, x_n)$n-phosphate.

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purified from human platelets and separated from an $InsP_6$ binding protein which was found to be vinculin [9]. An $InsP_4$ binding protein from mouse cerebellum which showed higher affinity for Ins(1,3,4,5,6)P_5 than Ins(1,3,4,5)P_4 was identified as synaptotagmin II [10]. From rat liver nuclei a 74 kDa high-affinity Ins(1,3,4,5)P_4 receptor was purified [11]. Besides an $InsP_6$ binding protein which was identified as clathrin assembly protein AP2 [12] two distinct Ins(1,3,4,5)P_4 binding protein complexes were purified fom rat cerebellum [13]. Recently a phosphatidylinositol 3,4,5-trisphosphate ($PtdPIP_3$) binding protein from rat brain was purified and molecularly cloned [14]. This 46 kDa protein was designated as centaurin-α and was shown to bind phosphatidylinositol 3,4,5-trisphosphate ($PtdPIP_3$) with higher affinity than Ins(1,3,4,5)P_4.

We have previously purified and characterized $p42^{IP4}$ an Ins(1,3,4,5)P_4 receptor from pig cerebellum which binds D-Ins(1,3,4,5)P_4 with high affinity [15]. The 42 kDa protein was identified by photoaffinity-labelling [16]. Immunological studies using an antipeptide antiserum suggested that this protein exists in a membrane-associtated and in a cytosolic form [17]. Here we investigate the binding specificity of this Ins(1,3,4,5)P_4 receptor protein using all $InsP_3$, $InsP_4$ and $InsP_5$ regioisomers (in the case of asymmetric regioisomers as racemic pairs) and some other inositolphosphates. The aim of this study was to identify the stereochemistry of the phosphate groups needed for high affinity binding to the purified receptor protein.

MATERIALS AND METHODS

Materials. D-[^{3}H]Ins(1,3,4,5)P_4 (770 GBq/mmol), Diphospho-myo-inositolpentakisphosphate (PP-$InsP_5$) and Bisdiphospho-myo-inositoltetrakisphosphate (PP-$InsP_4$-PP) were obtained from NEN DuPont (Bad Homburg, FRG), D-Ins(1,4,5)P_3 from BIOMOL (Hamburg, FRG), D-Ins(1,3,4,5)P_4 from CellSignal Inc. (Kingston RI, USA). The sodium salt of D-Ins(1,2,6)P_3 was provided by Perstorp Pharma (Lund, Sweden). D-GroPIP$_3$ was a gift from Dr. P. Cullen (Bristol, UK) and Mab Cl 41.1 from Dr. R. Jahn (New Haven, USA).

Synthesis of inositolphosphates. All regioisomers of $InsP_4$, $InsP_3$ and $InsP_5$ were synthesized by S.-K. Chung and coworkers, as previously described [18, 19, 20].

Receptor purification. The $InsP_4$ receptor protein was purified from pig cerebellum as described previously [15]. The last step of the purification protocol was modified by using the detergent LDAO (N,N-dimethyl-dodecylamine-N-oxide) instead of Brij 58. The purity of the fraction containing the receptor protein was checked by SDS-PAGE. Western blot using an antipeptide antiserum directed against a 19 amino acid peptide of the receptor protein allows to identify the 42 kDa protein [17]. In some experiments the monoclonal antibody Mab Cl 41.1 directed against the cytosolic fragment of synaptotagmin I [21] or Mab 46-1 directed against the myelin protein CNP (2′,3′-Cyclic Nucleotide 3′-phosphodiesterase; [22, 23]) was employed.

Binding assays. Binding of Ins(1,3,4,5)P_4 to the purified receptor protein was determined as described previously [15]. The $InsP_4$ receptor protein (50 to 250 ng) was assayed in 280 μl of buffer (25 mM sodium acetate/25 mM potassium phosphate, pH 5.0, 0.05 % Brij 58, 1 mM EDTA) supplemented with 0.8 to 1.1 nM [^{3}H]Ins(1,3,4,5)P_4 and varying concentrations of unlabelled D-Ins(1,3,4,5)P_4 or the other inositolphosphates tested. Nonspecific binding was determined in the presence of 1 μM D-Ins(1,3,4,5)P_4. After incubation for 20 min at 4°C, an aliquot of the reaction mixture was centrifuged through columns, containing 1.2 ml packed resin of BioGel P-4 (grade fine 65 μm; Bio-Rad) equilibrated with binding buffer, to separate bound from free ligand. 3 ml of scintillation fluid (Ultima Gold, Packard) were added for measuring the bound radioactivity. All determinations made in duplicate or triplicate showed less than 15 % deviations.

Data analysis. K_d values and the number of binding sites were estimated by using the RADLIG data-analysis computer program (Version 4; BIOSOFT, Cambridge, UK) as described previously [24]. In competition assays the data obtained with the displacing inositolphosphate were fitted to the data obtained for the displacement of [^{3}H]-Ins(1,3,4,5)P_4 by D-Ins(1,3,4,5)P_4 within the same set of experiments, i.e. K_d, B_{max} and non-specific binding were fixed to the values obtained from the homologous displacement curve.

RESULTS

We tried to analyze the characteristics of the binding site of the 42 kDa $InsP_4$ receptor protein. For the binding experiments we used a purified fraction in which the $InsP_4$ receptor was enriched by chromatography by a factor of at least 10,000. The samples of the $InsP_4$ receptor fraction obtained with our purification protocol contained the 42 kDa $InsP_4$ receptor

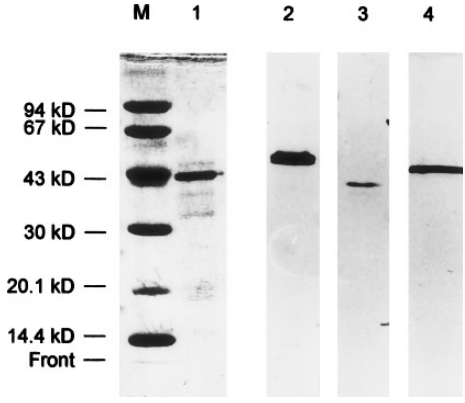

FIG. 1. SDS-PAGE and Western blot of the purified $InsP_4$ receptor fraction. The eluate obtained with 1 M NaCl from the hydroxyl apatite column during the purification of the $InsP_4$ receptor protein from pig cerebellum (lane 1: 1 μg, lane 2 and 3: 500 ng, lane 4: 50 ng of protein) as described [15, 16] was electrophoresed by SDS-PAGE. The proteins were either stained by coomassie blue R250 (lane 1) or transferred to a PVDF membrane and probed for the presence of the myelin protein CNP with the Mab 46-1 (lane 2, 0.8 μg/ml), the cytosolic fragment of synaptotagmin I with the MAb Cl 41.1 (lane 3, 4 μg/ml), and the $InsP_4$ receptor with an antipeptide antiserum (lane 4, 1 μg/ml affinity purified antibodies). The blots were developed by incubation with horseradish peroxidase conjugated antibodies (lane 2 and 3: goat anti mouse, lane 4: goat anti rabbit) followed by chloronaphtol/hydrogene peroxide.

protein which has been identified functionally by photoaffinity labeling [16] and in some cases traces of other proteins with molecular mass of 46, 37 and rarely another one with 32 kDa, as shown previously by SDS-PAGE ([16] and Fig. 1 lane 1). We identified two of these proteins by isolation and peptide sequencing. The 46 kDa protein was demonstrated to be the myelin protein CNP [22]. The 37 kDa protein turned out to be the cytosolic fragment of synaptotagmin I as demonstrated by the N-terminal sequence of the protein (Stricker and Reiser, unpublished data). The identity of the 37 kDa protein being the cytosolic fragment of synaptotagmin I was confirmed by Western blotting using the monoclonal antibody MAb Cl 41.1 [21]. In Fig. 1, lane 1 the SDS-PAGE of a fraction typically used for the binding assays described in the following section of this paper is shown. The identity of each, the $InsP_4$ receptor protein (lane 4), the CNP (lane 2) and the cytosolic part of synaptotagmin I (lane 3) is demonstrated by Western blotting. Fig. 1, moreover, shows that the 42 kDa protein is the major component in this fraction. In most cases the minor components could hardly be detected in the protein gel analysis. For immunological detection of the minor proteins in Western blot analysis a total amount of protein had to be applied which was at least 10 times higher than that needed to identify the $InsP_4$ receptor protein.

Binding assays were carried out with fractions similiar to that shown in Fig. 1. We employed all the trisphosphate, tetrakisphosphate and pentakisphosphate regioisomers of myo-inositol, the mesoforms and in the case of asymmetric regioisomers the racemic pairs. For some compounds the D-enantiomers were available. We also tested the water-soluble phosphatidyl-inositol (3,4,5)trisphosphate-analogue D-GroPIP_3.

The incubations were carried out in an acetate/phosphate buffer, pH 5.0 which was shown to yield highest binding of [^{3}H]Ins(1,3,4,5)P_4 as compared to the physiological buffer systems and to more alkaline pH values [24, 25]. Similar pH profiles for [^{3}H]Ins(1,3,4,5)P_4 binding were described for several other tissues, as in rat cerebellum [26], rat heart and brain [27], and canine smooth muscle [28]. Using an autoradiographic assay, Parent and Quirion [29] showed for rat and human brain the highest Ins(1,3,4,5)P_4 binding at pH 5.0. A striking advantage in using the acidic pH value is the clear discrimination between [^{3}H]Ins(1,3,4,5)P_4

TABLE 1
Displacement of D-[^{3}H]Ins(1,3,4,5)P_4 Binding to the Purified Receptor Protein by Different Inositoltetrakisphosphates

$InsP_4$	$K_i \pm \sigma_n$
D-Ins(1,3,4,5)P_4	2.20 ± 0.84 nM
D/L-Ins(1,3,4,5)P_4 = L/D-Ins(1,3,5,6)P_4	8.8 ± 1.5 nM
D/L-Ins(1,2,4,5)P_4 = L/D-Ins(2,3,5,6)P_4	143.4 ± 6.7 nM
D/L-Ins(1,2,4,6)P_4 = L/D-Ins(2,3,4,6)P_4	138.4 ± 23.3 nM
Ins(1,2,3,5)P_4	374.5 ± 29.2 nM
Ins(1,3,4,6)P_4	53.4 ± 14.3 nM
Ins(2,4,5,6)P_4	116.0 ± 7.3 nM
D/L-Ins(1,2,5,6)P_4 = L/D-Ins(2,3,4,5)P_4	268.3 ± 134.3 nM
D/L-Ins(1,2,3,4)P_4 = L/D-Ins(1,2,3,6)P_4	244.0 ± 47.4 nM
D/L-Ins(1,4,5,6)P_4 = L/D-Ins(3,4,5,6)P_4	54.6 ± 30.2 nM
D-GroPIP_3	1.33 ± 0.8 nM

Note. The assays were performed as described under Materials and Methods. The average B_{max}-value for five different receptor preparations used in the experiments was 10.4 ± 4.3 nmol/mg protein (n = 14). Non-specific binding was determined in the presence of 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 to 8 different concentrations. For D-Ins(1,3,4,5)P_4 the mean value was derived from 14 experiments, for the other compounds from 2 to 5 experiments.

and [^{3}H]Ins(1,4,5)P_3 binding sites. The latter show highest binding at alkaline pH values, where little or no $InsP_4$ binding is found [26, 28, 29].

Binding of [^{3}H]Ins(1,3,4,5)P_4 to the receptor protein was reversible and specific for D-Ins(1,3,4,5)P_4 as shown previously [30]. The K_d of the purified receptor protein for D-Ins(1,3,4,5)P_4 was 2.2 nM and the density of binding sites was 10.4 nmol/mg of protein (Table 1). The racemic mixture of D/L-Ins(1,3,4,5)P_4 inhibited binding of [^{3}H]Ins(1,3,4,5)P_4 with a K_i value of 8.8 nM indicating that the L-isomer of this compound does not bind to the receptor protein. All the other inositoltetrakisphosphate regioisomers tested were 25-to 150-fold less potent in displacing [^{3}H]Ins(1,3,4,5)P_4 than D-Ins(1,3,4,5)P_4 itself, whereas the compounds with a phosphate group at C-2 of the inositol ring were the weakest ligands in this group of inositolphosphates (Table 1). It is obvious from Table 1 that D-GroPIP_3 is as potent in displacing [^{3}H]Ins(1,3,4,5)P_4 from binding as unlabelled D-Ins(1,3,4,5)P_4 itself. The inositolphosphate head group of this compound resembles the structure of D-Ins(1,3,4,5)P_4. This raises the possibility that PtdIns(3,4,5)P_3 is a physiological ligand for the $InsP_4$ receptor protein.

Among the inositoltrisphosphates only D/L-Ins(1,5,6)P_3 displayed unique behaviour (see below). All other inositoltrisphosphates tested were more than three orders of magnitude less effective in displacing [^{3}H]Ins(1,3,4,5)P_4 than D-Ins(1,3,4,5)P_4 and D-GroPIP_3 (Table 2). As with the $InsP_4$ isomers the Ins(1,3,4,5)P_4 binding site does not tolerate a phosphate group at C-2 of the inositol ring of the trisphosphates. The physiologically important metabolite D-Ins(1,4,5)P_3 inhibits Ins(1,3,4,5)P_4 binding with a K_i higher than 10 μM thus distinguishing the $InsP_4$ receptor unequivocally from Ins(1,4,5)P_3 binding proteins. On the other hand D/L-Ins(1,4,5)P_3 shows a more than tenfold higher affinity for the $InsP_4$ receptor than the D-isomer of this compound. Since D-Ins(1,4,5)P_3 is L-Ins(3,5,6)P_3 and, on the other hand L-Ins(1,4,5)P_3 is identical with D-Ins(3,5,6)P_3 we conclude from the binding data that the compound D-Ins(3,5,6)P_3 is the displacing agent.

The racemic mixture D/L-Ins(1,5,6)P_3 displaced [^{3}H]Ins(1,3,4,5)P_4 with a more than tenfold

TABLE 2
Displacement of D-[^{3}H]Ins(1,3,4,5)P_4 Binding to the Purified Receptor Protein by Different Inositoltris- and -Pentakisphosphates

Ins P_x ($InsP_3$/$InsP_5$)	$K_i \pm \sigma_n$
Ins(1,2,3)P_3	> 10 μM
D/L-Ins(1,2,4)P_3 = L/D-Ins(2,3,6)P_3	> 10 μM
D/L-Ins(1,2,5)P_3 = L/D-Ins(2,3,5)P_3	> 10 μM
D/L-Ins(1,2,6)P_3 = L/D-Ins(2,3,4)P_3	12.9 $\pm$ 1.3 μM
D-Ins(1,2,6)P_3 = L-Ins(2,3,4)P_3	8.4 $\pm$ 0.8 μM
D/L-Ins(1,3,4)P_3 = L/D-Ins(1,3,6)P_3	2.6 $\pm$ 1.3 μM
Ins(1,3,5)P_3	> 10 μM
D/L-Ins(1,4,5)P_3 = L/D-Ins(3,5,6)P_3	1.23 $\pm$ 0.43 μM
D-ins(1,4,5)P_3 = L-Ins(3,5,6)P_3	> 10 μM
D/L-Ins(1,4,6)P_3 = L/D-Ins(3,4,6)P_3	5.4 $\pm$ 1.9 μM
D/L-Ins(1,5,6)P_3 = L/D-Ins(3,4,5)P_3	171.9 $\pm$ 90.6 nM
D/L-Ins(2,4,5)P_3 = L/D-Ins(2,5,6)P_3	> 10 μM
Ins(2,4,6)P_3	> 10 μM
Ins(4,5,6)P_3	5.4 $\pm$ 1.6 μM
3F-D-Ins(1,4,5)P_3	> 10 μM
D/L-Ins(1,2,4,5,6)P_5 = L/D-Ins(2,3,4,5,6)P_5	67.0 $\pm$ 9.3 nM
Ins(1,3,4,5,6)P_5	2.07 $\pm$ 0.97 nM
D/L-Ins(1,2,3,4,5)P_5 = L/D-Ins(1,2,3,5,6)P_5	20.6 $\pm$ 6.8 nM
Ins(1,2,3,4,6)P_5	446.4 $\pm$ 14.2 nM
PP-$InsP_5$	87.8 $\pm$ 44.7 nM
PP-$InsP_4$-PP	55.3 $\pm$ 25.4 nM

Note. Experimental details are given under Materials and Methods and legend to Table 1.

higher affinity than all the other $InsP_3$, regioisomers tested. As indicated in Table 2, this pair of enantiomers is identical to L/D-Ins(3,4,5)P_3, i.e. the D-enantiomer of Ins(1,5,6) is L-Ins(3,4,5)P_3 and the L-enantiomer of Ins(1,5,6)P_3 is D-Ins(3,4,5)P_3, respectively. Since the configuration of the phosphate groups at C-3, C-4- and C-5 of the D-Ins(3,4,5)P_3 molecule is the same as in the D-Ins(1,3,4,5)P_4 and D-Gro(3,4,5)PIP_3 molecules we conclude that D-Ins(3,4,5)P_3 (=L-Ins(1,5,6)P_3) is the displacing compound.

Using membranes from pig cerebellum Ins(1,4,5)P_3 and Ins(1,5,6)P_3 displayed the same effects in displacing [^{3}H]Ins(1,3,4,5)P_4 (Stricker and Reiser, unpublished data), as with the purified $InsP_4$ receptor protein shown here. 3-deoxy-3-fluoro-D-Ins(1,4,5)P_3 was characterized to bind to the $InsP_3$ receptor and to mobilize Ca^{2+} from intracellular stores, but not to be metabolized by the $InsP_3$ 3-kinase to D-Ins(1,3,4,5)P_4 [31]. This inositoltrisphosphate analogue showed no affinity for the $InsP_4$ receptor at concentrations of up to 10 μM, which is comparable to the data obtained with D-Ins(1,4,5)P_3 (Table 2).

For further characterization of the $InsP_4$ binding site all inositolpentakisphosphates (two mesoforms and two racemic pairs) were synthesized and used in displacement experiments. The results of these experiments are also summarized in Table 2. Ins(1,2,3,4,6)P_5 is the weakest ligand in displacing [^{3}H]Ins(1,3,4,5)P_4 from binding to the receptor protein (K_i= 446 nM). D/L-Ins(1,2,4,5,6)P_5 and D/L-Ins(1,2,3,4,5)P_5 displayed affinities in the range of 20 to 70 nM. Surprisingly, these affinities are higher than those found for most of the inositoltetrakisphosphates tested (Table 1). Ins(1,3,4,5,6)P_5 was even equipotent with D-Ins(1,3,4,5)P_4 in displacing [^{3}H]Ins(1,3,4,5)P_4 from receptor binding (2.07 nM versus 2.2 nM, respectively).

We previously reported that the affinity of the $InsP_4$ receptor for Ins(1,3,4,5,6)P_5 in mem-

branes was more than ten times lower than the value found for D-Ins(1,3,4,5)P_4. After solubilization of the receptor protein by detergent from the membrane fraction the affinity for Ins(1,3,4,5,6)P_5 was increased 10-fold [15]. Similarly the affinity of the receptor for D/L-Ins(1,2,4,5,6)P_5 was increased by a factor of 5 to 10 when the protein was solubilized with detergent from membranes, whereas there was no effect of solubilization and purification on the K_i for D/L-Ins(1,2,3,4,5)P_5 (data for affinities of the membrane-bound receptor are not shown here).

The diphosphoinositol polyphosphates PP-InsP_5 and PP-InsP_4-PP [32] displaced [^{3}H]-Ins(1,3,4,5)P_4 from the purified receptor protein with affinities in the range of 50 to 100 nM (Table 2). However, these compounds are only poor displacers of [^{3}H]Ins(1,3,4,5)P_4 when tested with membranes from pig cerebellum displaying K_i-values higher than 1 μM (data not shown). InsP_6 which has already been tested previously [15, 16] inhibited binding of D-Ins(1,3,4,5)P_4 with a K_i value of at least 10 μM.

DISCUSSION

We show here the ligand specificity of a previously identified InsP_4 receptor by testing all possible InsP_3, InsP_4 and InsP_5 regioisomers (asymmetric compounds as racemic mixtures) synthesized as described [18, 19, 20]. From some of the asymmetric regioisomers also the stereochemically pure D-isomers, were used as well as D-GroPIP_3 and the diphosphoinositol polyphosphates PP-InsP_5 and PP-InsP_4-PP. Among all the compounds tested we found three isomers which displaced nearly equipotently [^{3}H]Ins(1,3,4,5)P_4 with high affinity from the purified receptor. These were D-Ins(1,3,4,5)P_4, D-GroPIP_3 and Ins(1,3,4,5,6)P_5 (Table 1 and 2). All the other regioisomers of inositolphosphates showed potencies in displacement of [^{3}H]Ins(1,3,4,5)P_4 from binding to the receptor which were at least 10 times lower than the values for these three compounds.

In membranes from pig cerebellum D-Ins(1,3,4,5)P_4 and D-GroPIP_3 also show the same high affinity (not shown). However, the affinity of Ins(1,3,4,5,6)P_5 is at least tenfold lower with the receptor in membranes than with the detergent solubilized receptor protein [15]. The reason for the difference in selectivity for Ins(1,3,4,5,6)P_5 versus Ins(1,3,4,5)P_4 may due to the presence of detergent rather than to the removal of membrane lipids, since we recently found that the receptor protein exists also in a cytosolic, soluble form. This cytosolic protein has the same ligand affinity as the membrane associated protein for Ins(1,3,4,5,6)P_5 (not shown).

The binding data given in Tables 1 and 2 allow the following conclusions about the ligand binding requirements of p42^{IP4}. The substitution of the hydroxyl at C-2 of the inositol ring by a phosphate group is not tolerated by the binding site. Phosphate groups at C-3, C-4 and C-5 of the inositol ring are required for high affinity binding (compare D/L-Ins(1,4,5)P_3 and D/L-Ins(3,4,5)P_3). This is also highlighted by the fact that phosphorylation at C-1, C-3 and C-4 or at C-1, C-3 and C-5 does not yield a high-affinity ligand but that phosphorylation at C-3, C-4 and C-5 or the additional phosphorylation of C-4 or C-5 in Ins(1,3,5)P_3 or D/L-Ins(1,3,4)P_3, respectively, enhances the ligand affinity.

The phosphorylation of the hydroxyl at C-1 increases the affinity of p42^{IP4} for inositolphosphates which are phosphorylated at C-3, C-4 and C-5 (compare D/L-Ins(1,3,4,5)P_3 and D/L-Ins(3,4,5)P_3 or D/L-Ins(3,4,5,6)P_4 and Ins(1,3,4,5,6)P_4). Further substitution at C-1, i.e. esterification of the phosphate group with the glycerol moiety in D-GroPIP_3 does not diminish but seems to increase the affinity of the ligand for the binding site (Table 1). Previously we had reported the synthesis and usage of a photoaffinity analogue of D-Ins(1,3,4,5)P_4 in which the phosphate group at C-1 of the inositol ring was coupled to an N-(4-azidosalicyl)aminoethanol moiety. This modification reduced the ability of this compound to displace D-[^{3}H]Ins(1,3,4,5)P_4 from receptor binding by a factor of three [16]. Investigations with phosphatidylinositol 3,4,5-

trisphosphate derivatives which have additional fatty acids esterified to the glycerin moiety may give more insight whether these compounds are possible endogenous ligands for the $InsP_4$-receptor.

For the 104 kDa Ins(1,3,4,5)P_4 binding protein purified from porcine platelets a specificity different from that reported here was described [33]. The binding site on the 104 kDa protein was shown to be highly selective for D-Ins(1,3,4,5)P_4 and to recognize primarily the phosphates at C-1, C-3 and C-5, whereas a phosphate group at C-4 and C-6 had only little influence on binding. A phosphate group at C-2 was not tolerated [33] as with the 42 kDa $InsP_4$ receptor from pig brain.

The relatively high affinities of the inositolpentakisphosphates and of the diphosphoinositol polyphosphates seen here could be due to the high negative charge of these molecules interacting with the basic $InsP_4$ receptor molecule at the binding assay conditions [15, 30].

The receptor preparations used throughout the experiments described here were shown to contain, beside the 42 kDa $InsP_4$ receptor protein (Fig. 1 lane 1 and 4) traces of some contaminating proteins [15]. Two of these proteins could be identified, the 46 kDa protein as the myelin protein CNP [22] and the 37 kDa protein as the cytosolic fragment of synaptotagmin I (Fig. 1 lane 2 and 3). The cytosolic fragment of synaptotagmin I contains two C2-domains from which the C2B-domain was reported to bind Ins(1,3,4,5)P_4 and other inositolpolyphosphates [34]. Interestingly this domain was also found to bind Ins(1,3,4,5)P_4 in the $GAP1^{IP4BP}$ from pig platelet [8]. C2B-domains are present in different proteins, for example synaptotagmin I-IV, rabphilin 3A, protein kinase Cα, GTPase activating proteins (GAP), but inositolphosphate binding is only found in those C2B-domain containing proteins which possess a cluster of consecutive lysin residues [8, 34]. A sequence homologous to this inositolphosphate binding motive from C2B-domains was recently described for centaurin-α, a possible PIP_3 receptor protein from rat brain [14].

The following findings exclude that the inositolphosphate binding measured in the experiments described in this paper could be partly due to the contaminating synaptotagmin I fragment. The B_{max} values found for the receptor preparations (approx. 10 nmol/mg protein) are much too high to correlate with the trace amount of the synaptotagmin I fragment in this fraction (compare Fig. 1, lane 1, lane 3 and lane 4). Photoaffinity labelling with N-(4-azidosalicyl)aminoethanol(1)-1-phospho-D-myo-inositol 3,4,5-trisphosphate using a receptor fraction comparable to that in Fig. 1 resulted in the specific labelling of the 42 kDa protein [16] but not the 37 kDa protein. Another striking point is the difference in specificity and affinity for Ins(1,3,4,5)P_4 and other inositolphosphates between synaptotagmin C2B-domains and the $InsP_4$-receptor described here (compare [34], and Table 1 and 2).

Furthermore, using our purification scheme [15] the synaptotagmin I fragment was highly enriched during the purification of the $InsP_4$-receptor from pig cerebellum in a specific fraction (the eluate obtained with buffer containing 500 mM NaCl from a heparin agarose column). Binding assays using this eluate yielded no significant specific Ins(1,3,4,5)P_4 binding. Therefore, we can conclude that the binding measured in the experiments described here is due to Ins(1,3,4,5)P_4 binding to the 42 kDa $InsP_4$ receptor protein.

The property of the 42 kDa $InsP_4$ receptor $p42^{IP4}$ to bind $GroPIP_3$ with an affinity comparable to that for Ins(1,3,4,5)P_4 is also found with centaurin-α [14]. Centaurin-α, a protein of 46 kDa, binds both PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4 with, however, moderate affinities (IC_{50} values of 120 and 620 nM for PIP_3 and Ins(1,3,4,5)P_4, respectively). The absolute values of the affinities reported for these two proteins differ by more than two orders of magnitude. One possible reason for this discrepancy is the fact that Hammonds-Odie and coworkers made their determinations under non-equilibrium conditions i.e. by photoaffinity labelling. Surprisingly with [^{3}H]Ins(1,3,4,5)P_4 they could not detect reversible binding. In contrast to this, D-$GroPIP_3$ is only a weak ligand when tested with the $GAP1^{IP4BP}$ displaying a ten times lower affinity

than D-Ins(1,3,4,5)P_4 [7]. Molecular cloning of centaurin-α [14] showed that the peptide sequence identically contains peptide-3 from the 42 kDa $InsP_4$ receptor from pig cerebellum which we used previously for the generation of the antipeptide antiserum [17]. Using this antiserum we found in Western blots of membranes from different parts of the rat brain a protein comigrating in SDS-PAGE with the 42 kDa protein from pig cerebellum [17], but not a protein from rat brain at 46 kDa (cf. Fig. 4 in [17]). Thus, the 42 kDa $InsP_4$-receptor $p42^{IP4}$ and centaurin-α are closely related isoforms of $InsP_4$- / PIP_3-binding proteins.

D/L-Ins(3,4,5,6)P_4 which is identical to L/D-Ins(1,4,5,6)P_4 displaced [^{3}H]Ins(1,3,4,5)P_4 from the 42 kDa $InsP_4$ receptor from pig cerebellum with a K_i of 55 nM (Table 1). This compound was also the strongest displacer when tested with the $GAP1^{IP4BP}$ from pig platelets and further analysis showed that the $GAP1^{IP4BP}$ had slightly higher affinity for D-Ins(1,4,5,6)P_4/L-Ins(3,4,5,6)P_4 than for D-Ins(3,4,5,6)P_4/L-Ins(1,4,5,6)P_4 [33]. In contrast to these results the D-Ins(3,4,5,6)P_4 isomer displaced [^{3}H]Ins(1,3,4,5)P_4 from binding to the purified Ins(1,3,4,5)P_4 receptor from rat liver nuclei with an IC_{50} of 90 nM [11]. From our data presented above, i.e. the requirement of phosphate groups at C-3, C-4 and C-5 for high affinity binding we conclude that at the 42 kDa $InsP_4$ receptor from pig cerebellum, D-Ins (3,4,5,6)P_4/L-Ins(1,4,5,6)P_4 is the displacing compound. Both compounds, D-Ins(3,4,5,6)P_4 and D-Ins(1,4,5,6)P_4, were postulated to be possible second messengers in physiological processes ([35, 36] and the references therein).

Here we made an in-depth analysis of the specificity of the binding site of the 42 kDa $InsP_4$ receptor. This is important for further experiments delineating the properties of the binding pocket of the protein $p42^{IP4}$. Furthermore, our study using all regioisomers underpins the possibly unique role of the 42 kDa protein in the cell physiology, since all the inositolpolyphosphates possibly formed upon hormonal stimulation besides Ins(1,4,5)P_3, e.g. Ins(3,4,5,6)P_4 have a significantly lower affinity for this protein. Physiological effects of Ins(3,4,5,6)P_4 reported [36, 37] are mediated via completely different ligand recognition sites which can be deduced from the affinity profiles [36, 37] which are not overlapping with the one described here.

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