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PEPTIDE-SPECIFIC ANTIBODIES INDICATE SPECIES HETEROGENEITY OF A 42 kDa HIGH-AFFINITY INOSITOL 1,3,4,5-TETRAKISPHOSPHATE RECEPTOR PROTEIN FROM BRAIN

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Summary: The aim of the present study was to identify a high affinity $InsP_4$ receptor in membranes from cerebellum or brain from several species. In samples obtained from sheep, beef, human and rat, a 42 kDa protein represents an $Ins(1,3,4,5)P_4$ receptor, similar to the $InsP_4$ receptor from pig cerebellum, which we have described previously using an $InsP_4$-photoaffinity analogue (Reiser et al., Biochem. J. 1991, 280, 533). CNBr cleavage of the pig receptor protein for peptide sequencing revealed peptide sequences which demonstrate that the 42 kDa $InsP_4$ receptor is a novel protein. Two synthetic peptides derived from the pig receptor were used to generate peptide-specific antisera which recognized also the intact receptor protein from pig. The two antisera showed different reactivity with the $InsP_4$ receptor purified from pig and human compared to sheep, beef, and rat. This indicates a species heterogeneity of this protein.

The role of $InsP_3$ ($Ins(1,4,5)P_3$) in intracellular signal transduction has been well established [1]. $InsP_3$ formed after activation of various hormone receptors binds to receptors which have an inbuilt Ca^{2+}-release channel located on intracellular organelles which form part of the endoplasmic reticulum [2]. $InsP_3$ is either inactivated by a phosphatase or phosphorylated [3] generating $InsP_4$ ($Ins(1,3,4,5)P_4$). The role of $InsP_4$ in Ca^{2+} regulation and in cellular signalling is still unclear [4].

$InsP_4$ has been proposed to cause re-sequestration of Ca^{2+} into intracellular stores [5] or to activate Ca^{2+} release from internal stores [6]. Activation of Ca^{2+}-permeable ion channels by $InsP_4$

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Abbreviations used: $InsP_4$, $Ins(1,3,4,5)P_4$, Inositol(1,3,4,5)tetrakisphosphate; LDAO, N, N-Dimethylamine-dodecylamine-N-oxide; ELISA, enzyme linked immunosorbent assay.

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has been measured in membrane patches of cultured bovine endothelial cells [7]. In ras-transformed fibroblasts it has been shown that $InsP_4$ induces Ca^{2+} influx [8]. The controversy about the mode and site of action of $InsP_4$ could be resolved by identifying the receptor protein for $InsP_4$.

An $InsP_4$ receptor has been established as a high-affinity binding site for radioactive $InsP_4$ in membranes [9, 10, 11]. We have purified a high affinity $InsP_4$ receptor from pig cerebellar membranes as a protein with apparent molecular mass of 42 kDa [12]. Employing a photoaffinity label, we functionally identified the 42 kDa protein as the $InsP_4$ receptor [13, 14]. Theibert et al. [15] reported that solubilization by treatment of rat brain membranes with CHAPS and subsequent chromatography yielded an elution profile with two different peaks of $InsP_4$-binding activity. Samples from these peaks contained various protein complexes. Using a photoaffinity analogue of $InsP_4$ comparable to that described in our previous study, Theibert et al. [16] observed labelling of several proteins, which also included an $InsP_6$ receptor. An $InsP_4$/ $InsP_6$ receptor has been identified as the clathrin-assembly protein AP-2 [17,18]. This $InsP_6$ receptor has been found to have K^+ channel activity [18]. Cullen et al. [19] have used the purification scheme developed for isolating he 42 kDa receptor protein by Reiser et al. [20] to search for such receptors in pig platelet membranes and found a protein with apparent molecular mass of 104 kDa. Thus, in non-neural tissues there could be a different type of $InsP_4$ receptor.

However, it still has to be ascertained that an $InsP_4$ receptor is responsible for physiological responses of this putative second messenger. Here we investigate which protein corresponds to the $InsP_4$-binding activity in membranes prepared from cerebellum and cortex from several different species. Furthermore two anti-peptide antisera have been raised against peptides which were synthesized according to the sequences obtained from CNBr cleavage products of the $InsP_4$ receptor from pig. The antisera served as a tool to investigate differences of the $InsP_4$ receptor in different species.

Materials and Methods

Solubilization and purification of the Ins(1,3,4,5)P_4 receptor. Preparation of membranes from cerebella or from brain cortex and solubilization of proteins in buffer containing the detergent Brij 58 (1.5% w/v) were carried out as described [12]. Buffer A contained (mM): Tris-HCl (50), EDTA (1), mercaptoethanol (1), pH 7.5, and Brij 58 (0.1% w/v). The solubilized sample was centrifuged (1 h, 100,000 x g), the resulting supernatant dialyzed in buffer A without detergent, and applied to a CM-cellulose column equilibrated with buffer A. Proteins were eluted by buffer A with NaCl concentration steps of 50 mM and 0.2 M. The $InsP_4$ binding activity was found in the fractions eluted with 0.2 M NaCl. These were pooled, dialyzed in buffer A without detergent and applied to a heparin-agarose column equilibrated with buffer A. This column was eluted by increasing the NaCl concentration discontinuously to 0.4, 0.5 and 0.8 M. The fractions of the 0.8 M NaCl eluate with $InsP_4$ binding activity were pooled, dialyzed in buffer B and subjected to hydroxylapatite chromatography. Buffer B contained (mM) KH_2PO_4/ K_2HPO_4 (25), EDTA (1), mercaptoethanol (1), pH 7.5, and LDAO (0.1% w/v). The fractions eluted from the hydroxlyapatite column with buffer B supplemented with 1 M NaCl showed $InsP_4$ binding activity. These were pooled and used for analysis. Binding of [^{3}H]$InsP_4$ and displacement by non-radioactive $InsP_4$ (0.5 - 500 nM) was assayed in solubilized and purified preparations by spun-column chromatography [12].

Peptide sequencing. Sequence analysis was performed with peptides from the $InsP_4$ receptor purified from pig cerebellum. For these experiments the proteins in the eluate obtained from the hydroxylapaptite column were precipitated with acetone/methanol (1:1) and then separated by

SDS PAGE. The protein band of 42 kDa molecular mass was excised from the gel and digested with CNBr (35 mg/ml in 70% formic acid) for 15 h at room temperature. The supernatant containing the peptides was dried in a vacuum centrifuge (Bachofer, FRG), dissolved in sample buffer and applied to gel electrophoresis with a 16.5% acrylamide gel [21]. The peptides were transferred to PALL membranes by semi-dry blotting. Peptide bands stained with Coomassie blue were cut out and sequenced, using a gas phase sequencer (Applied Biosystems).

Immunological procedures. Peptides used for immunization were synthesized by continuous flow solid-phase peptide synthesis using a MilliGen (Eschborn, Germany) 9050 synthesizer based on Fmoc/Bu^t strategy. The peptides were purified by semipreparative HPLC and their identity was tested by ion spray mass spectrometry (data no shown). Peptides were coupled either to KLH (keyhole limpet haemocyanin, Calbiochem) or to bovine serum albumin (Sigma) with 7 mM glutaraldehyde (1 mg peptide per 2 mg carrier protein) as described in Harlow and Lane [22].

Antisera were raised in rabbits (Chinchilla bastards) by multiple subcutaneous injections of the immunogen. For the first immunization, 0.5 ml conjugate (0.7 mg of peptide for peptide-1 and 1 mg of peptide for peptide-2) emulsified in an equal volume of complete Freunds adjuvant (Gibco BRL) were injected. For boost immunizations we injected conjugate with 0.2 mg peptide-1 or 0.7 mg peptide-2 in Freunds adjuvant (equal volumes of complete and incomplete adjuvants) in a total volume of 1 ml at 4 weeks intervals. The rabbits were bled from an ear vein 8-12 days after each boost injection. The titer of the antisera was determined with an ELISA. The ELISA and Western blot were carried out as described [23]. Protein contents of detergent-containing samples were measured by Amido Black. The materials used have been described [20, 23].

Results and Discussion

We made the attempt to isolate the $InsP_4$ receptor present in membranes of various species, i.e. sheep, beef, human and rat. Solubilized material obtained from the membranes was chromatographed following the protocol used for purification of a high-affinity $InsP_4$ receptor from pig cerebellar membranes [20]. Fig. 1A shows a SDS/PAGE analysis of the fractions obtained during chromatography of solubilized membranes from sheep cerbellum. In membranes solubilized with Brij 58 (lane 1) $InsP_4$ binding activity was 22 fmol/mg protein. After CM-chromatography we obtained maximal $InsP_4$ binding activity of 104 fmol/mg protein (fraction applied in lane 2). In the following heparin-agarose-chromatography substantial $InsP_4$ binding activity (4060 fmol/mg protein) was found only in the fraction eluted by buffer with high NaCl concentration (lane 7). This fraction was passed through a hydroxylapaptite column. The maximal $InsP_4$ binding activity of 7000 fmol/ mg protein was seen in the eluate obtained with buffer containing 1 M NaCl. This fraction showed one most prominent protein band with an apparent molecular mass of 42 kDa in SDS-PAGE (Fig. 1A, lane 8). The second fraction from the hydroxylapaptite column, in which several additional proteins could be detected with molecular masses of 46 kDa, 37 kDa, 32 kDa and some other smaller proteins (Fig. 1A, lane 9), had a substantially lower specific $InsP_4$ binding activity. The values for $InsP_4$ binding activity serve to assess the degree of purification of the $InsP_4$ receptor and do not represent the maximal binding capacity, which can be derived from Scatchard analysis only.

Thus, in sheep cerebellum a 42 kDa protein represents the $InsP_4$ receptor, as previously seen with pig cerebellum [20]. Solubilized preparations of membranes from human brain, from beef cerebellum, and from rat brain were subjected to the same chromatography. In all experiments a 42 kDa protein band was the predominant protein in the fraction with the maximal $InsP_4$ binding activity (data not shown). Binding of [^{3}H]$InsP_4$ to the receptor isolated from sheep cerebellum is half-maximally displaced by $InsP_4$ at 10 nM concentration (Fig. 1B). Scatchard analysis of binding

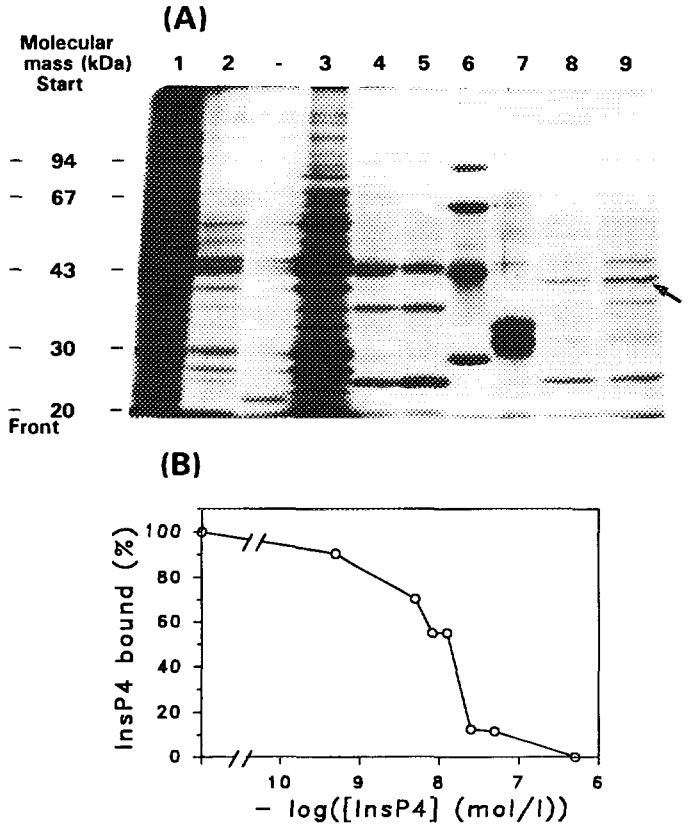

Fig. 1. Characteristics of $InsP_4$ receptor protein from sheep cerebellum. (A) SDS PAGE analysis of fractions obtained during chromatographic purification and (B) binding of [^{3}H]Ins(1,3,4,5)P_4 to the high-affinity Ins-P_4 receptor. **(A)** Lane 1: Cerebellar membranes solubilized with Brij 58 (150 µg of protein); lane 2: Fraction eluted by 0.2 M NaCl from the CM-cellulose (37 µg of protein); lane 3: Fraction eluted by 0.4 M NaCl from heparin-agarose column (240 µg); lane 4 and 5: Fractions eluted by 0.5 M NaCl from heparin-agarose column (12 µg); lane 7: Approximately 8 µg of protein eluted by 0.8 M NaCl from heparin-agarose column; lane 8: First fraction (8 µg) eluted from the hydroxylapaptite column by 1M NaCl, and lane 9: Second fraction eluted from the hydroxylapaptite column by 1M NaCl; lane 6: Molecular mass markers [20]. Sample preparation and electrophoresis of proteins as described [20]. Gel stained firstly with Coomassie brilliant blue R250 and afterwards with silver. **(B)** Displacement of binding of [^{3}H]Ins(1,3,4,5)P_4 by unlabelled Ins(1,3,4,5)P_4. The receptor protein was purified by CM-cellulose, heparin-agarose, and hydroxylapaptite chromatography (sample shown in A, lane 8). Binding was determined according to Donié et al. [12] with 2 µg of protein in the assay in duplicates. 100% specific binding corresponds to 11 fmol Ins(1,3,4,5)P_4 bound at an [^{3}H]$InsP_4$ concentration of 0.2 nM. Comparable results were obtained in 2 experiments. Specific binding is total binding minus unspecific binding, which was determined in the presence of 0.5 µM non-radioactive Ins(1,3,4,5)P_4.

experiments using samples comparable to that shown in Fig. 1 A, lane 8, yielded a dissociation constant of 11 nM and a maximal binding capacity of 385 pmol/ mg of protein. Previously we have found comparable properties for the $InsP_4$ receptor isolated from pig cerebellar membranes [20].

We tried to further characterize the $InsP_4$ receptor by determining peptide sequences. Attempts to obtain an amino acid sequence from the purified pig $InsP_4$ receptor were not successful. This indicates that the amino terminus of the protein was blocked. Therefore the 42 kDa protein

band, which appeared after separation by SDS PAGE, was excised from the gel and treated with cyanogen bromide. The resulting peptide mixture was analyzed by PAGE and resolved into peptide fragments of the following molecular masses: (1) 13 kDa, (2) 8 kDa, (3) 7.5 kDa, (4) 7 kDa, (5) 5 kDa, and (6) 4 kDa. Since fragment (2) could not be sequenced this peptide may contain the blocked amino terminus. For fragment (1) we obtained the following amino acid sequence: H-Ala-Ser-Arg-Gly-Asn-Ser-Ala-Ala-Arg-Val-Phe-Glu-Ser-Arg-Val-Pro-Pro-Phe-Tyr-Tyr-OH. For fragment (5) the sequence was: H-Lys-Ile-Glu-His-Leu-Asn-Ala-Thr-Phe-Gln-Pro-Ala-Lys-Ile-Gly-OH. In fragment (5) there were ambiguities at positions 16 and 18 and then another 7 amino acid residues could be identified. Amino acid sequences from both fragment (1) and fragment (5) cannot be found in protein databases (PIR, Swissprot) nor do they exhibit any significant homology with sequences in known proteins. Therefore we can conclude that the high affinity $InsP_4$ receptor from brain, which we have isolated, represents a novel protein.

For the generation of antipeptide antisera peptide-1 and peptide-2 were synthesized, coupled to bovine serum albumin or to KLH as carrier proteins and injected into rabbits. The immune response was determined by ELISA. The antibody titer of the sera which is defined as the dilution giving an absorbance of 1 was after the first boost immunization with peptide-1 or peptide-2 1:2.500 and 10.000, respectively. These values are derived from ELISA's in which the wells of the plates were coated with peptide-1 and -2. Coating the wells with a fraction containing the receptor protein (cf. lane 9 in Fig. 1A), gave a sizeable ELISA signal only after the second boost immunization.

The antisera were also tested in Western blots to find out whether the antibodies recognized the 42 kDa $InsP_4$ receptor. The SDS PAGE in Fig. 2A shows the fractions obtained during a purification of the receptor protein from pig cerebellar membranes. The pattern of protein bands in the fractions from the chromatography corresponds to that seen in Fig 1A. The 42 kDa protein band is marked by an arrow in Fig. 2A. The gel shown in Fig 2A was subjected to a Western blot (Fig. 2B) using antiserum against peptide-2. In lane 3, where the fraction containing maximal $InsP_4$ binding activity in the first chromatographic step (CM cellulose) is applied, there is a weak reaction with one protein band. The fraction with maximal $InsP_4$ binding activity from the second column (heparin-agarose) which is applied in lane 7 shows a strong immunoreactivity at 42 kDa. A similarly intensive reaction was obtained with the final fraction from the hydroxylapaptite column (Fig. 2, lane 9). Antiserum against peptide-2, even at dilutions of 1:1000, detected the 42 kDa $InsP_4$ receptor in Western blots. For these experiments samples purified by chromatography on CM-, heparin- and hydroxylapaptite- columns with about 5 µg of total protein were applied to the gel electrophoresis.

We employed the antisera against peptide-1 and peptide-2 to probe the $InsP_4$ receptor from various species. In the Western blots in Fig. 3 (lanes 1-5) identical samples are tested with antiserum against peptide-2 (A) or against peptide-1 (B). The $InsP_4$ receptor from pig cerebellum in lanes 4 and 5 (about 6 and 1.5 µg of protein, respectively) is recognized by both antisera at dilutions of 1:50. The $InsP_4$ receptor from sheep cerebellum, however, is recognized by antipeptide-2 antiserum but not by antipeptide-1 serum (Fig.3, lanes 1- 3; for SDS PAGE analysis, cf. Fig. 1A, lane 8). The $InsP_4$ receptor from beef cerebellum gives a positive signal with serum against peptide-2 (Fig. 3A, lanes 6-8). An identical blot with the same samples did not react with antipeptide-1 antiserum (not shown). $InsP_4$ receptor from rat brain, human brain, and also from pig cerebral cortex was

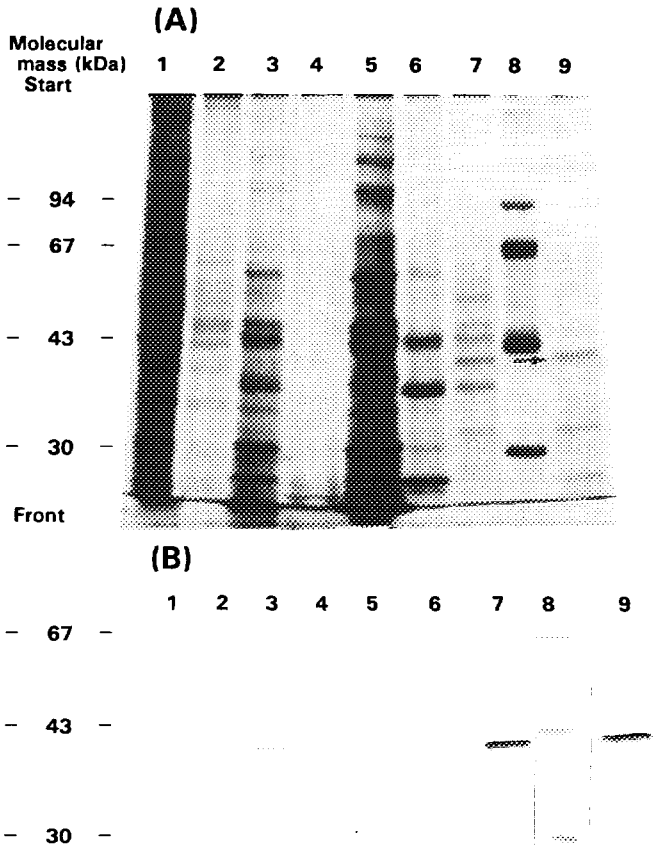

Fig. 2. Identification of $InsP_4$ receptor protein from pig cerebellum by anti-peptide antiserum. (A) SDS PAGE analysis and (B) Western blot with identical samples obtained from fractions obtained during purification of the high-affinity Ins-P_4 receptor. Lane 1: Cerebellar membranes solubilized with Brij 58; lanes 2, 3 and 4: fractions eluted by 0.05, 0.2, and 0.8 M NaCl from the CM-cellulose; lanes 5, 6, and 7: fractions eluted by 0.4, 0.5, and 0.8 M NaCl from heparin-agarose column; lane 9: Fraction eluted by 1 M NaCl from the hydroxylapaptite column; lane 8: Molecular mass markers. The 42 kDa protein is marked by an arrow. Electrophoresis in (A) was caried out in 10% polyacrylamide gel (3% polyacrylamide stacking gel), stained firstly with Coomassie brilliant blue R250 and afterwards with silver. The Western blot in (B) was developed with antiserum against peptide-2 (dilution 1:70).

immunopositive in Western blots probed with antiserum against peptide-2. The limit of detection was about 0.5 μg of protein applied to the gel (Fig. 3, lane 3).

Thus, the high-affinity $InsP_4$ receptor of 42 kDa molecular mass can be found in several species. However, antipeptide antisera indicate some species heterogeneity because antiserum against peptide-1 was positive with the protein from pig (Fig. 3 B), human and rat brain (not shown), but did not react with the receptor from sheep and beef. The antiserum, which was specific for peptide-2, gave a comparably strong signal with receptor from pig, human, beef, sheep and rat. Clarification of the primary structure of the $InsP_4$ receptor will answer the question whether peptide-1 is absent or modified in the protein from beef or sheep.

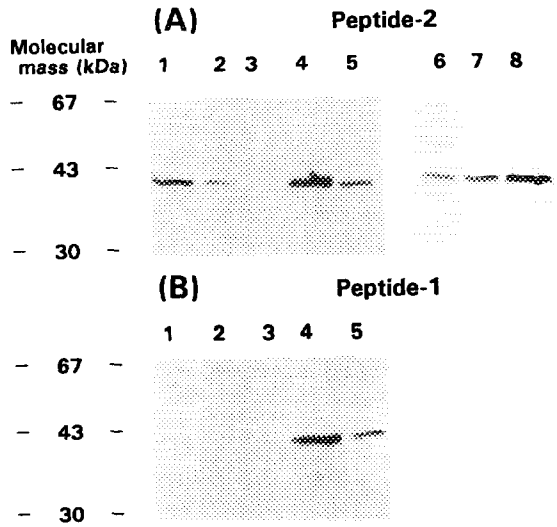

Fig. 3. Antipeptide antisera show species specificity of the 42 kDa $InsP_4$ receptor protein. Western blot analysis with antiserum against peptide-2 (A) or peptide-1 (B) at a dilution of 1:50 of samples subjected to SDS PAGE and to electrophoretic transfer to PVDF membranes. In A and B, lanes 1 - 5, identical samples were used. Lanes 1, 2, and 3: 8, 2 and 0.4 µg of $InsP_4$ receptor protein purified from sheep cerebellum. Lanes 4 and 5: Approx. 6 and 1.5 µg of $InsP_4$ receptor protein purified from pig cerebellum. In A, lanes 6- 8, $InsP_4$ receptor from beef cerebellum probed with antiserum against peptide-2 with fractions obtained from heparin-agarose (lane 6) or from hydroxylapatite (lanes 7 and 8) with about 8 µg of total protein was applied to the gel.

The two antisera for the $InsP_4$ receptor protein did not recognize the protein in membrane samples from brain where the amount of the $InsP_4$ binding activity is rather low [9]. Therefore analysis of further pig tissues for the presence of the 42 kDa protein could not be carried out. For that reason immunoprecipitation experiments using these antisera were also not possible. Apart from the species heterogeneity indicated, there may also be different types of $InsP_4$ receptors, as already described for the $InsP_3$ receptors [24]. Furthermore, the antipeptide antisera presented here should also be useful for immunohistochemical analysis to localize the $InsP_4$ receptor.

Recently an Ins 1,3,4,5 P_4 binding protein isolated from mouse cerebellar membranes has been identified by partial amino acid sequencing as synaptotagmin II [25]. However, in our purification scheme of the $InsP_4$ receptor from pig cerebellum synaptotagmin is not copurified with the 42 kDa protein [Stricker and Reiser, unpublished observation]. Therefore elucidation of the molecular identity of the 42 kDa protein will allow identification of a novel type of $InsP_4$ receptor.

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