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Characterization of a high-affinity Ins-P_4 (inositol 1,3,4,5-tetrakisphosphate) receptor from brain by an anti-peptide antiserum

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Abstract From a high-affinity Ins-P_4 (inositol 1,3,4,5-P_4) receptor purified from pig cerebellum, digested with the protease Lys C peptide sequences were obtained. Synthetic peptide-3 (19 amino acid residues) was used to generate an antiserum. Reaction of the affinity-purified antibodies with the purified pig receptor protein in ELISA or Western blot was completely inhibited by peptide-3. In cerebellar membranes, the antibodies clearly recognized the 42 kDa Ins-P_4 receptor protein and two additional proteins (25 kDa, 37 kDa) which still have to be identified. The anti-peptide antibodies could selectively immunoprecipitate the Ins-P_4 receptor protein. The antiserum was used (i) to demonstrate that in brain from different species (human, pig, beef, rat, mouse and sheep) a similar 42 kDa Ins-P_4 receptor protein is contained, and (ii) to obtain indications for the existence of a related soluble form of the 42 kDa Ins-P_4 receptor besides the membrane-associated receptor.

Key words: Inositolphosphate; Signal transduction; Affinity purification

1. Introduction

The second messenger Ins(1,4,5)P_3 (D-myo-inositol 1,4,5-trisphosphate) triggers the release of Ca^{2+} from internal stores [1]. The physiological role of Ins(1,4,5)P_3 has been clearly established by purifying [2] and functionally reconstituting the high-affinity Ins(1,4,5)P_3 receptor [3]. The involvement of Ins(1,3,4,5)P_4 (D-myo-inositol 1,3,4,5-tetrakisphosphate), a phosphorylation product of Ins(1,4,5)P_3 [4], in cellular Ca^{2+} regulation is not yet understood. This issue can be resolved only by identifying the Ins-P_4 receptor proteins.

Previously we have described a receptor with high affinity for Ins(1,3,4,5)P_4 in cerebellar membranes [5]. The receptor protein could be solubilized from porcine cerebellar membranes, purified and identified by photoaffinity labeling as a protein with an apparent molecular mass of 42 kDa in SDS-PAGE [6]. From the porcine receptor protein we obtained peptide sequences after cleavage of the protein by treatment with CNBr or with the protease Lys C (Hülser, Lottspeich, Hoppe, and Reiser, unpublished) and synthesized some of these peptides. Here we describe the characteristics of an anti-peptide antiserum raised against one of these peptides (peptide-3) and its application for characterizing the receptor protein.

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Abbreviations: Ins-P_4, Ins(1,3,4,5)P_4, D-myo inositol 1,3,4,5-tetrakisphosphate; KLH, keyhole limpet hemocyanine; PVDF, polyvinylidene difluoride

2. Methods

2.1. Purification of the Ins(1,3,4,5)P_4 receptor

Membrane preparation from pig cerebellum was carried out as previously described [5,7]. In some experiments the buffers used contained protease inhibitors (1 mM benzamidine, 0.2 mM Pefabloc SC, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM EGTA). Membranes from beef cerebellum, sheep cerebellum and porcine brain cortex were prepared by using the same protocol. The Ins-P_4 receptor protein from pig cerebellum was solubilized and purified as described [7,8].

2.2. Preparation and purification of a peptide-3 specific antiserum

Peptide-3 (19 amino acid residues) was synthesized (Kalbacher; data not shown) and coupled to keyhole limpet hemocyanine (1 mg peptide per 2 mg KLH) for immunization [9]. For the first immunization an equal volume of complete Freund's adjuvant was added to the peptide-3–KLH-conjugate (0.6 mg) and the emulsion was injected subcutaneously into a rabbit (female Chinchilla bastard). Subsequent immunizations were carried out with a mixture of 0.2 mg conjugate in incomplete Freund's adjuvant. The rabbit was bled 10 days after immunization and serum was tested for antibody response by ELISA and Western blot using the preimmune serum as control. The peptide-3 specific antibodies were purified from the antiserum by protein A sepharose chromatography [9], followed by chromatography with peptide-3 coupled to AFFI-Gel 10. For synthesis of the affinity resin peptide-3 (33 mg dissolved in 1.5 ml DMSO) was incubated with AFFI-Gel 10 (1.4 ml packed resin) for 2 h at room temperature. Remaining activated ester groups were blocked with 50 mM ethanolamine, pH 8.0. The affinity resin was poured into a column (Ø 1 cm) and washed with PBS containing 500 mM NaCl. The eluate from the protein A-sepharose chromatography was dialysed against PBS, adjusted to 500 mM NaCl, and applied to the affinity column (12 ml/h). After washing with 4 sample volumes of 500 mM NaCl/PBS the bound antibodies were eluted with 100 mM glycine/NaOH pH 2.5.

2.3. ELISA

ELISA assays were carried out as described previously [10]. In competitive ELISA tests the antiserum was preincubated with different concentrations of peptide-3 (40 min, room temperature) and then used as primary antibodies. The plates were then further incubated with horse radish peroxidase conjugated IgG goat anti-rabbit and developed.

2.4. SDS-PAGE and immunoblotting

SDS-PAGE was carried out with 3% stacking gel and 10 or 12.5% separation gels according to Laemmli. The gels were stained either with Coomassie brilliant blue or with silver. For identification of antigen by Western blot the proteins were transferred to PVDF membranes by electroblotting and developed as described [10,11].

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2.5. Immunoprecipitation of the Ins-P_4 receptor protein by peptide-3 specific antibodies

A fraction enriched in the Ins-P_4 receptor protein which was obtained by CM-cellulose chromatography [5] was incubated overnight at 4°C in the presence of either (i) 185 nM purified anti-peptide-3 antibodies and 5 mg protein A sepharose, or (ii) 185 nM purified anti-peptide-3 antibodies, 20 μM peptide-3 and 5 mg protein A sepharose, or (iii) 5 mg protein A sepharose (control), in a total volume of 1.15 ml of Tris-Buffer (50 mM TRIS/HCl, pH 7.4, 1 mM mercaptoethanol, 1 mM EDTA, supplemented with 0.1% BRIJ 58 and 500 mM NaCl). The protein A-sepharose immunoglobulin complexes were pelleted by centrifugation (11,000 × *g*, 25 min, 4°C), and the pellets were washed twice with Tris-buffer by resuspension and centrifugation. The antigen–antibody complexes were eluted from the protein A sepharose beads by addition of 300 μl 100 mM glycine/NaOH, pH 2.5. After centrifugation the supernatants were neutralized with 30 μl 1 M TRIS/HCl, pH 8.0 and analyzed by SDS-PAGE and Western blot.

3. Results and discussion

A high-affinity inositol 1,3,4,5-tetrakisphosphate receptor was purified from pig cerebellum using the purification scheme established previously in our laboratory [5,7]. The protein was digested with Lys C, and the peptides were purified by HPLC [11]. Peptide sequencing yielded some partial amino acid sequences which showed no homologies to any known proteins which are retrievable in current data bases (Hülser and Lottspeich, unpublished). In order to get more information about the Ins 1,3,4,5-P_4-binding protein one of the peptides, designated peptide-3 was synthesized (data not shown). Peptide-3 which has 19 amino acid residues was the longest among these peptides with the amino acid sequence KVVDRPMLPQEYAVEAHFK.

Coupling of the synthetic peptide to keyhole limpet hemocyanine and subsequent immunization of a rabbit with this conjugate gave an antiserum which recognized the peptide and the purified receptor protein in an ELISA. Using ELISA plates coated with 20 ng of peptide-3, the titer of the antiserum, defined as the dilution yielding an absorbance of 1.0 according to our ELISA protocol used [11] was about 1 : 100,000. In Western blot analysis, the purified receptor protein was detected specifically by this antiserum (data not shown). In all these experiments, preimmune serum was used as a control and did not show any response to the antigenes.

Immunoglobulins were purified from the serum using protein A-sepharose chromatography followed by affinity chromatography with the peptide-3 coupled to an AFFI-Gel 10 resin. In a typical purification run protein A-sepharose chromatography gave 4.3 mg immunoglobulins from 1 ml serum from which the following affinity chromatography extracted 0.34 mg specific anti-peptide-3 antibodies. With the second purification step we could remove most of the KLH-specific immunoglobulin IgG which occurred at a high titer in the antiserum.

The purified anti-peptide antibodies were used for ELISA and Western blot tests (Fig. 1). Fig 1A shows the results from a competitive ELISA. In this experiment, the antiserum was preincubated with varying concentrations of peptide-3 and then used for the ELISA reaction in wells coated with different amounts of peptide-3 or of the receptor protein. Fig. 1B gives an indication of the affinity of peptide-3 antiserum for the Ins-P_4 receptor after gel electrophoresis and transfer to PVDF membranes. The immunoreactivity is nearly completely suppressed when the antiserum has been pretreated with 4 μM peptide. Thus, the antibodies show a high affinity for the protein, whether in native (Fig. 1A) or in partially denatured form (Fig. 1B). Moreover, binding of the peptide antibodies to the purified receptor protein is specific since it could be displaced by the peptide. In control experiments the preimmune serum did not recognize the antigen (data not shown).

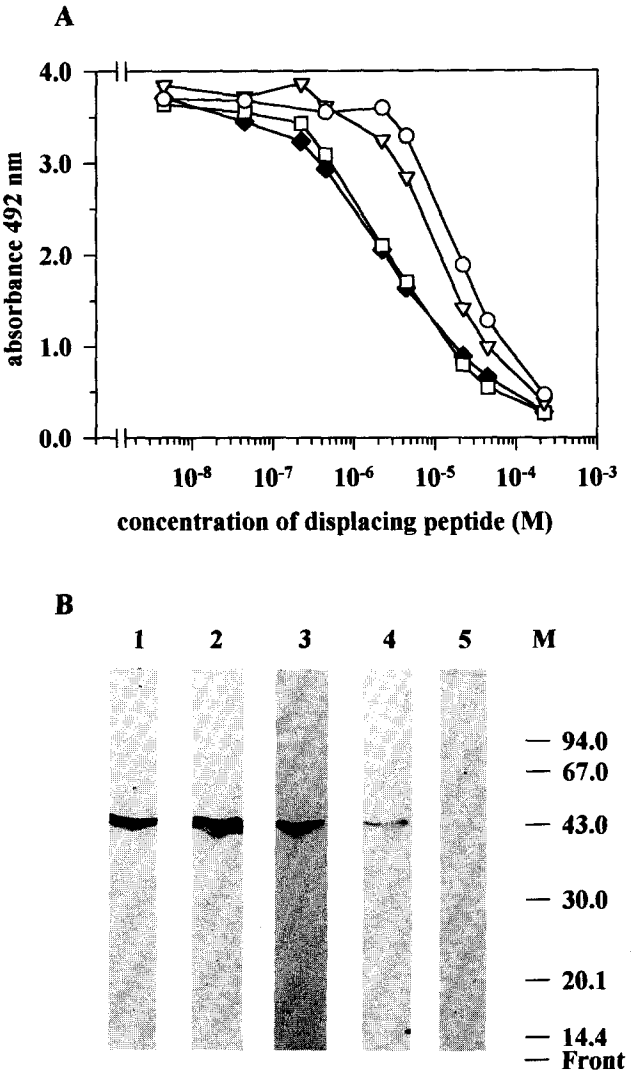

Fig. 1. Peptide-3 displaces anti-peptide-3 antibodies from binding to the purified Ins-P_4 receptor protein in ELISA (A) in Western blot (B). (A) Competitive ELISA with synthetic peptide-3 used as competing antigen. The ELISA plates were coated with different amounts of peptide-3 (○ 7.5, ▽ 5, and □ 2.5 ng/well) or purified Ins-P_4 receptor protein (◆, approx. 10 ng/well). Purified peptide-3 specific antibodies (0.5 nM) were preincubated with peptide-3 at the concentrations indicated (0–220 μM). (B) Immunoblot. SDS- PAGE (12.5% separation gel) and blot of purified receptor protein (approx. 1 μg per lane) onto PVDF-membranes. The blots were incubated with purified peptide-3 specific antibodies (lane 1: 4.8 nM, lanes 2 to 5: 24 nM), in the presence of varying concentrations of peptide-3 (lane 3: 0.44 μM, lane 4: 4.4 μM, lane 5: 44 μM). M: Molecular mass standards.

Specific staining of the Ins-P_4 receptor by the purified anti-peptide antiserum is demonstrated in Fig. 2, where SDS-PAGE analysis (A) of various samples is shown in parallel with the corresponding Western blots (B). Even in the crude membrane

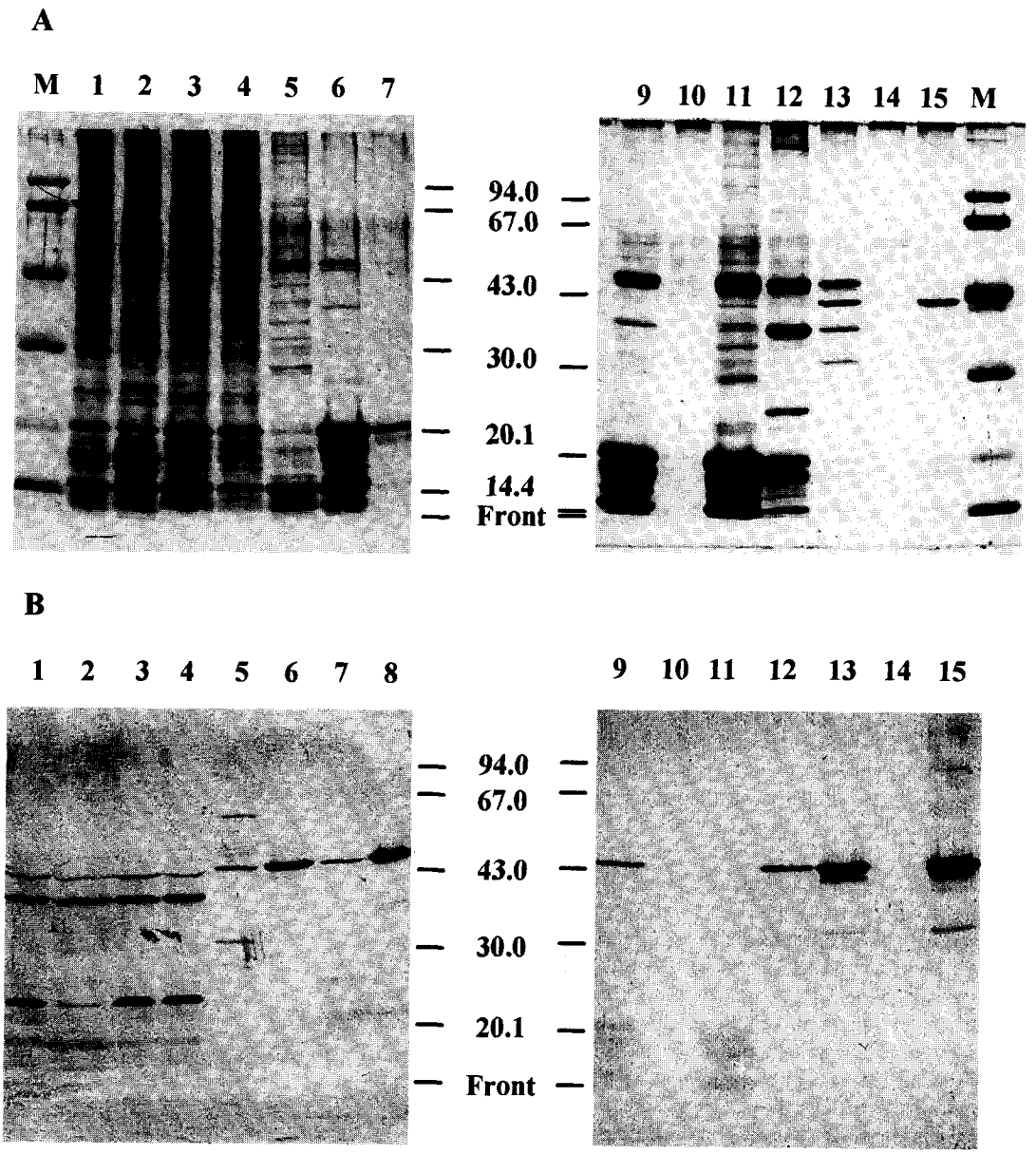

Fig. 2. SDS-PAGE (A) and Western blot analysis (B) of the purification of the Ins-P_4 receptor from pig cerebellum [5]. Lanes 1 to 7: Solubilization and CM-cellulose chromatography: Membranes from pig cerebellum (lane 1); the pellet (lane 2) and the supernatant (lane 3) after solubilization of the membranes and centrifugation (100,000 × *g*); CM-cellulose column flow-through (lane 4), and fractions eluted with 50 mM NaCl (lane 5), 200 mM NaCl (lane 6) and 800 mM NaCl (lane 7). Lane 8 (B only): Control sample of purified Ins-P_4 receptor (100 ng). Lanes 1 to 4, 40 μg (A) and 100 μg (B), lanes 5 to 7, 20 μg (A) and 40 μg (B) of protein. Lanes 9 to 15: Heparin-agarose and hydroxyapatite chromatography. Fraction loaded on the heparin-agarose column (lane 9; 10 μg); flow-through (lane 10) and the fractions eluted with 400 mM NaCl (lane 11, 10μg), 500 mM NaCl (lane 12, 8 μg) and 800 mM NaCl (lane 13, 3 μg) from the heparin-agarose column. The latter fraction was loaded on a hydroxyapatite column. Flow-through (lane 14) and fraction eluted with 1 M NaCl from the hydroxyapatite column (lane 15, 3μg). Gel (12.5% separation gel) was stained with Coomassie blue (A, lanes 1 to 7) and with silver (A, lane 9 to 15). The blots (B) were developed with the purified anti-peptide-3 antibodies (21 nM). M: Molecular mass standards.

preparation from pig cerebellum (Fig. 2, lane 1), the 42 kDa band is recognized by the antiserum. Two additional protein bands with approximate molecular mass of 37 kDa and 25 kDa are also recognized. Reaction with the 37 kDa and 25 kDa proteins is inhibited by peptide-3 (not shown).From the solubilized proteins contained in the 100,000 × supernatant, in the Western blot (Fig. 2B, lane 3), the 42 kDa protein band and the two smaller proteins were stained. In the insoluble material from the 100,000 × *g* pellet the 25 kDa protein band was only a minor constituent (Fig. 2B, lane 2).

As already previously demonstrated, the Ins-P_4 receptor protein is retained by CM cellulose at pH 7.5. However, the two smaller proteins detected by the anti-peptide-3 antiserum within the membranes and the solubilized proteins do not bind to CM cellulose and, thus, can be found only in the flow-through (Fig. 2B, lane 4). In the fractions eluted from the CM

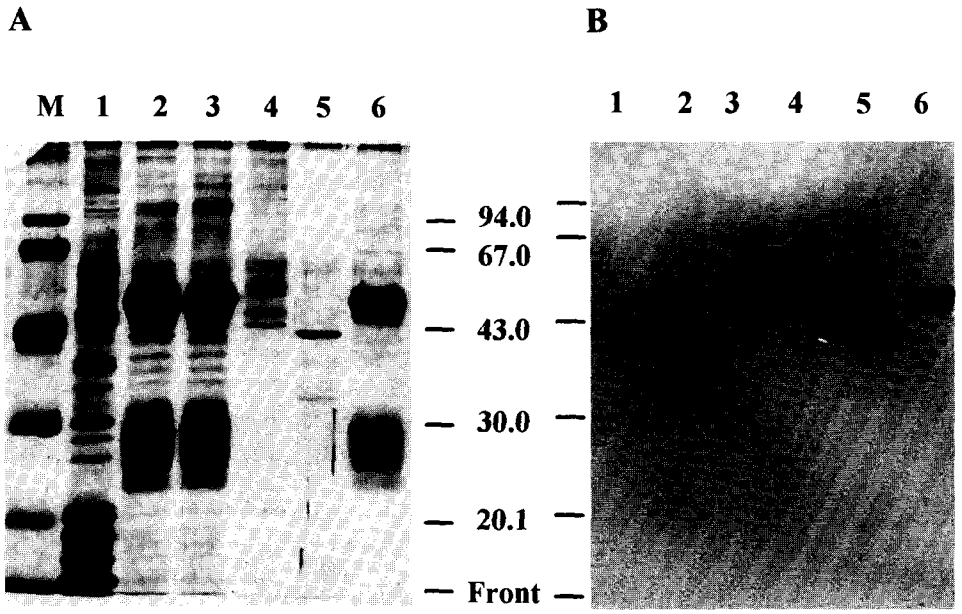

Fig. 3. Immunoprecipitation of the Ins-P_4 receptor protein by peptide-3 specific antibodies. Analysis of samples by SDS-PAGE and silver staining of the gel (A, 12.5% separation gel), and by immunoblot (B). Lane 1: Sample applied to the immunoprecipitation (28 μg protein, cf. Fig. 2, lane 6); lane 2: immunoprecipitation of the Ins-P_4 receptor (150 μl of the eluate); lane 3: inhibition of immunoprecipitation of the receptor by peptide-3 (150 μl of the eluate); lane 4: control experiment (150 μl of the eluate); lane 5: purified Ins-P_4 receptor (1.5 μg protein); lane 6: purified peptide-3 specific antibodies (3.1 μg protein); M: molecular mass standards. The blot in B was incubated with peptide-3 specific antibodies (21 nM).

cellulose by stepwise increasing the NaCl concentration of the buffer (lanes 5, 6, and 7), the highest amount of 42 kDa protein was found at 200 mM NaCl (lane 6). When this fraction (Fig. 2, lane 9) was subjected to heparin agarose chromatography, most of the proteins were retained by the column which can be seen in the analysis of flow-through (lane 10). The elution profile obtained with stepwise increasing the NaCl concentration (lanes 11, 12, and 13) is consistent with binding data. Ins-P_4 binding is maximal in the fraction eluted by 800 mM NaCl buffer [5] where maximal immunoreactivity was detected (lane 13). The final, apparently homogeneous fraction which was the result of the third chromatography used (hydroxyapatite) strongly reacted with the antiserum (cf. also the experiment depicted in Fig. 1B). During the chromatographic purification of the Ins-P_4 receptor the amount of immunoreactivity (42 kDa protein band) is enriched in those fractions in which Ins-P_4 binding is maximal [5].

The capacity of the anti-peptide antibodies to immunoprecipitate the receptor protein was tested. Fig. 3 shows the SDS-PAGE analysis of the immunoprecipitation. For this experiment a fraction enriched in the receptor protein (lane 1) which was obtained from the CM-cellulose chromatography (eluted with 200 mM NaCl; cf. Fig. 2A, lane 6) was incubated with antiserum and then with protein A beads. In the precipitate (lane 2) the 42 kDa protein is clearly seen in the silver-stained SDS gel below the immunoglobulin heavy chain. No precipitation occurs in the presence of peptide-3 (lane 3). The precipitation by the antiserum is further proven in Fig. 3B, where the SDS gel shown in A has been probed in a Western blot. The precipitated protein detected in lane 2 is clearly not found in lane 3. In lane 5 a sample of the purified Ins-P_4 receptor protein was applied and in lane 6, for comparison, purified peptide-3-specific antiserum has been run on the gel.

In further experiments, cerebellar membranes or brain homogenates from various species were analyzed. The Western blot in Fig. 4A shows that in all species examined (rat, mouse, human, sheep and beef) the antiserum detected the 42 kDa protein. Immunoreactivity of the smaller proteins recognized by the antiserum was unequally distributed among different species. The 25 kDa protein showed the highest intensity in the homogenate from human cerebellum (Fig 4A, lane 5), but was barely detectable in rat and mouse (lanes 1 and 2). The 37 kDa protein could not be detected by the antiserum in mouse brain or human cerebellum.

The fractions obtained during preparation of membranes from pig cerebellum were analyzed using the anti-peptide antiserum (Fig. 4B). The antiserum recognizes the 42 kDa Ins-P_4 receptor in the homogenate (lane 1). Most surprisingly, in the fraction containing mainly cytosol which is obtained after centrifugation (35,000 × g), a 42 kDa protein band which was also present in two further wash solutions whether supplemented with 0.4 M NaCl (lane 4) or not (lane 6) was detected by the anti-peptide-3 antiserum. The 37 kDa protein band only appeared in the membrane pellets derived from the centrifugation (lanes 3, 5, and 7) whereas the 25 kDa protein band was enriched in the soluble fractions. The membranes were prepared in the presence of a cocktail of protease inhibitors (see section 2.1). Thus, we can rule out the possibility that the 42 kDa protein is a proteolytic fragment of a larger precursor protein. Similarly, the smaller proteins which are recognized by the antiserum are most likely not proteolytic fragments of the 42 kDa protein.

Here we have shown that an anti-peptide antiserum raised with a 19 amino acid residue fragment derived from the 42 kDa high affinity Ins-P_4 receptor specifically recognizes the intact protein. Specificity is demonstrated by the fact that the antise-

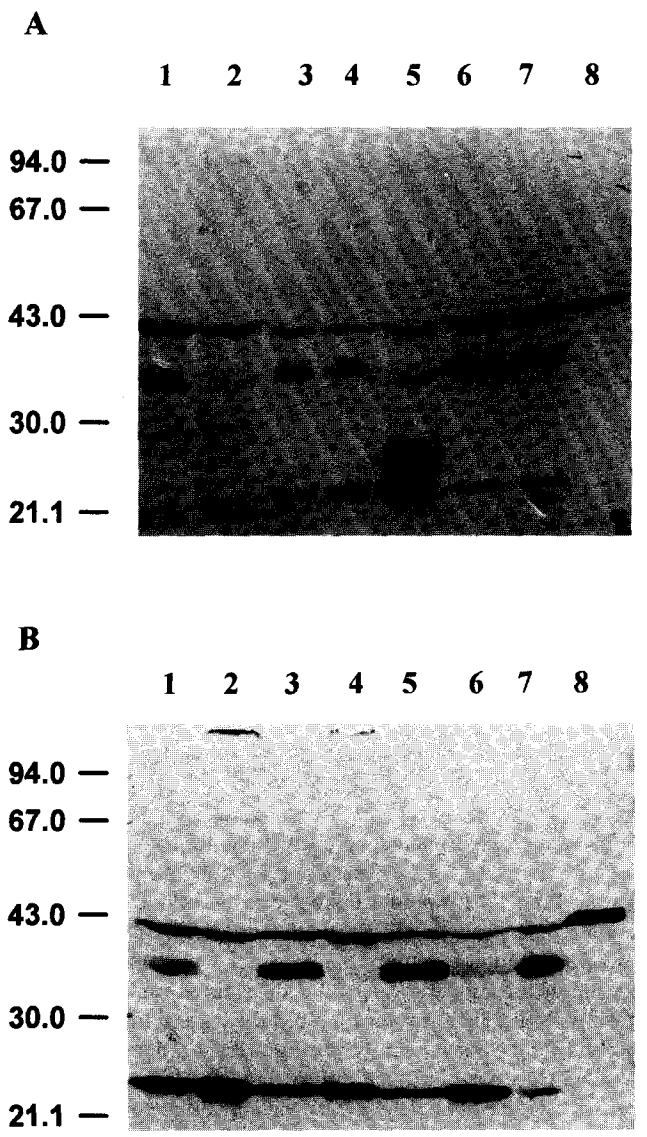

Fig. 4. Distribution of the Ins(1,3,4,5)P_4 receptor protein in brain tissue from different species (A) and in the fractions obtained during preparation of membranes from pig cerebellum (B). A: Homogenates from rat cortex (lane 1), mouse whole brain (lane 2), human cerebellum (lane 5), and membranes from sheep cerebellum (lane 3), beef cerebellum (lane 4), pig cortex (lane 6) and pig cerebellum (lane 7). B: The fractions which are obtained during preparation of membranes from pig cerebellum. Lane 1: homogenate from pig cerebellum; lane 2: supernatant with mainly cytosolic fraction from pig cerebellum; lane 3: crude membranes; lane 4: 0.4 M NaCl wash fraction (20,000 × *g* supernatant); lane 5: salt-washed membranes; lane 6: second wash fraction (without NaCl), and lane 7: final membrane preparation. The proteins (100 μg in each lane) were separated by SDS-PAGE (10% separation gel) and blotted onto PVDF-membranes. The blots were developed with the peptide-specific antibodies (21 nM). Lane 8 (A and B): A fraction enriched in the Ins(1,3,4,5)P_4 receptor protein from pig cerebellum (comparable to Fig. 2, lane 6, 14 μg protein) shown as control.

rum reaction is blocked by the peptide. The question of the identity of the two additional proteins recognized by the antiserum (37 and 25 kDa) seems to be an interesting issue. They are most likely not proteolytic fragments of the 42 kDa receptor protein. Moreover the cellular distribution of these proteins is not matching. The 25 kDa protein is found at a large quantity in the soluble fraction (Fig. 4) associated with the salt-washed membranes, solubilized by detergent and only to a very small degree occurring in the insoluble material, i.e. cytoskeleton and cellular matrix (Fig. 2, lane 2). The 37 kDa protein associated with membranes does not appear in a soluble form.

Both the 25 kDa and 37 kDa proteins have in common, in contrast to the Ins-P_4 receptor, not to be retained by the cation exchanger CM cellulose. This indicates the presence of a smaller positive net charge than in the Ins-P_4 receptor. The identity of these proteins with a species-specific distribution pattern (Fig. 4A) still has to be unravelled.

The putative second messenger Ins-P_4 might well act by several functionally related proteins. Previously Theibert et al [12] have described protein complexes occurring in brain tissue which bind Ins-P_4 and with similar affinity Ins-P_5. One of these proteins displays K^+ channel activity [13]. The recently reported 104 kDa protein with Ins-P_4 binding activity found in pig platelets [14] suggests the possibility that in non-brain, peripheral tissue Ins-P_4 acts via some other Ins-P_4 receptor proteins. Consistent with these interpretations we find in Western blot analysis of membranes from peripheral pig tissues (liver, heart, spleen and kidney) that the peptide-3 antibodies do not recognize a 42 kDa protein. The characteristics of the 42 kDa Ins-P_4 receptor described here will enable us to propose hypotheses regarding the cellular function of this protein which will give insight into the physiological role of Ins-P_4.

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