Trypsin digestion of the inositol trisphosphate receptor: implications for the conformation and domain organization of the protein

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Limited digestion of rat cerebellum microsomal vesicles with trypsin resulted in the proteolysis of the 240 kDa inositol 1,4,5-trisphosphate receptor (IP₃R) and the formation of a 94 kDa species that remained membrane-bound and retained immuno-reactivity to an antibody raised against the C-terminal sequence of this protein. The appearance of the 94 kDa species was associated with a loss of [³H]IP₃ binding sites in the membrane and the appearance of [³H]IP₃ binding sites in the soluble fraction. The 94 kDa fragment retained reactivity to biotinylated concanavalin A. *In vitro* phosphorylation of the IP₃R in membranes with cyclic AMP-dependent protein kinase and [γ -³²P]ATP produced an unlabelled 94 kDa fragment after tryptic digestion. According to current models of the cerebellar IP₃R this would place the proteolytic site between the phosphorylation

site at serine-1755 and the first transmembrane segment of the IP₃R. A second antibody raised to amino acids 401–414 in the N-terminal region of the receptor recognizes a 68 kDa fragment released into the soluble fraction after trypsin treatment. The time course of release of the 68 kDa fragment was correlated with the appearance of soluble binding sites, and the fragment was bound by IP₃-Affigel resin. A large proportion of the 68 kDa fragment remained associated with the membrane fraction and could be specifically immunoprecipitated from detergent extracts of digested membranes by anti-C-terminus antibody. Our results provide experimental evidence to further localize the ligand binding domain and suggest that regions of the N-terminus and C-terminus may be non-covalently associated.

INTRODUCTION

Intracellular Ca²⁺ mobilization in response to many hormones, growth factors and neurotransmitters is mediated by the release of inositol 1,4,5-trisphosphate (IP₃) [1]. This molecule interacts with a specific receptor glycoprotein (IP₃R) that has been purified from several sources [2–5]. The IP₃R is tetrameric, with a subunit molecular mass on SDS/PAGE of 240–260 kDa. Functional reconstitution of the purified protein in liposomes [6,7] and planar lipid bilayers [8] indicates that the IP₃R is itself a ligand-gated Ca²⁺ channel.

Large amounts of the type-I IP₃R protein are found in the Purkinje neuron of the cerebellum [9]. The full-length cDNA encoding the type-I IP₃R isoform has been cloned from mouse [10], rat [11], *Drosophila* [12] and *Xenopus* [13]. Mutational analysis [14,15] and experiments with photo-activatable IP₃ analogues [16] have suggested that the ligand binding domain of the receptor is located in the N-terminal 650 amino acids. The major portion of the receptor faces the cytoplasmic space and is believed to contain a regulatory domain, including phosphorylation sites and sites for ATP binding. *In vitro*, the brain IP₃R is a substrate for protein kinase A (A-kinase) [17], protein kinase C [18] and Ca²⁺/calmodulin-dependent protein kinase II [18]. The location of the phosphorylation sites for A-kinase have been mapped to serine residues 1589 and 1755 [19].

There is growing evidence that multiple forms of the IP₃R may exist in different tissues. Two alternatively spliced variants of the IP₃R have been reported. One has a 15-amino-acid deletion in the N-terminal region and has been detected in brain [11,20]. The other, which appears to be the predominant form in vas deferens and fetal brain, has a 40-amino-acid deletion between the A-kinase phosphorylation sites [21]. Recently, two additional IP₃

receptors have been cloned. The type-II IP₃R is predominantly found in brain, lacks the A-kinase sites and has 65% sequence identity with the type-I IP₃R [14]. The type-III IP₃R is found predominantly in pancreatic islets, kidney and the gastrointestinal tract [22,23]. This isoform has consensus sites for several protein kinases and has 62% sequence identity with the type-I receptor. Partial sequence data for a fourth form of IP₃R have also been reported [24].

The C-terminal region of the protein contains several stretches of hydrophobic residues that could potentially span the membrane. However, the transmembrane topology of the receptor is controversial [25]. Based on hydropathy analysis, models with six [12,26,27], seven [10], eight [11,14,22] or nine [10] transmembrane loops have been proposed. In an effort to obtain further information on the domain structure and transmembrane topology of this protein, we have used limited proteolysis of the IP₃R in brain microsomal membranes and anti-IP₃R antibody, ligand and lectin binding to study the proteolytic fragments. We provide experimental evidence that further localizes the ligand binding domain of the receptor and suggests that portions of the N-terminus and C-terminus may be non-covalently associated. Our experimental results are also incompatible with some models of IP₃R topology.

MATERIALS AND METHODS

Proteolysis of membranes

Microsomal membranes were prepared from rat cerebellum homogenates by differential centrifugation as described previously [28]. Routinely, digestion of membranes was carried out at 1 mg of protein/ml in trypsin treatment buffer containing

Abbreviations used: IP₃, myo-inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; cAMP, cyclic AMP; ConA, concanavalin A; A-kinase, cAMP-dependent protein kinase; HRP, horseradish peroxidase; DTT, dithiothreitol.

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120 mM KCl, 20 mM Tris/HCl (pH 7.8), 1 mM EDTA, 1 mM dithiothreitol (DTT) and 10–20 $\mu\text{g}/\text{ml}$ trypsin (Boehringer Mannheim). After incubation at room temperature for 5 min, a 10-fold excess of soybean trypsin inhibitor (Sigma Chemical Co.) and 1 mM PMSF were added. Membranes and soluble fractions were recovered by centrifugation at 100000 g on a Beckman Benchtop ultracentrifuge using the TLA 100 rotor. When required, the IP_3Rs in control or trypsin-digested membranes were solubilized (1 mg of protein/ml) on ice for 30 min using 1% (w/v) Triton X-100 and a buffer containing 0.32 M sucrose, 20 mM Tris/HCl, pH 7.8, 1 mM DTT and 0.5 mM PMSF (solubilization buffer). This buffer was additionally supplemented with 5 $\mu\text{l}/\text{ml}$ of a proteinase-inhibitor cocktail stock solution which contained aprotinin (0.6 mg/ml), pepstatin A (0.45 mg/ml), leupeptin (0.3 mg/ml), soybean trypsin inhibitor (0.2 mg/ml) and antipain (0.45 mg/ml). Detergent extracts were obtained after centrifugation for 45 min at 100000 g .

Immunoprecipitation and immunoblotting of the IP_3R

A polyclonal antibody was raised in a rabbit to a synthesized peptide corresponding to the C-terminus of the type-I IP_3R (residues 2731–2749; [10,11]) which was coupled via an additional N-terminal cysteine to keyhole limpet haemocyanin using the bifunctional cross-linking agent *N*-succinylimidyl 3-(2-pyridyl-dithio)propionate [29]. The characteristics of this antibody and experimental conditions for immunoprecipitation and immunoblotting have been described [30]. Briefly, immunoprecipitation was carried out in 100 μg aliquots of Triton X-100-solubilized extracts in a final volume of 1 ml of solubilization buffer supplemented with 0.1% SDS. The samples were incubated for 2 h with 100 μg of immune serum, and immune complexes were precipitated after a further 2 h incubation with 100 μl of 20% (v/v) Protein A–Sepharose. After washing three times in immunoprecipitation buffer, the beads were resuspended in 50 μl of SDS gel sample buffer boiled for 5 min, and samples were analysed on SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose sheets and processed with antibodies using the following sequential steps interposed by three 5 min washes with Tris-buffered saline containing 0.05% (v/v) Tween-20 (TTBS): blocking for 2 h with 3% (w/v) BSA in TTBS; incubation for 1 h with a 1:500 dilution of immune or preimmune serum; incubation for 1 h with a 1:3000 dilution of biotinylated goat anti-rabbit IgG (Vector Labs); incubation for 30 min with a 1:3000 dilution of horseradish peroxidase (HRP)–avidin D (Vector Labs) and colour development with 4-chloro-1-naphthol as described in [31]. In some experiments immunoreactive bands were detected with ^{125}I -labelled goat anti-rabbit IgG (ICN Radiochemicals) or an enhanced chemiluminescence kit (Amersham Corp.).

A second anti- IP_3R antibody used in this study (referred to as KEEK-Ab) was raised against a peptide corresponding to amino acids 401–414 of the type-I receptor. The procedures used for coupling and preparation of the antibody were the same as described for the anti-C-terminus antibody. The antibody was purified on a Protein A–agarose column [31] and then affinity-purified using the peptide coupled to EMPHAZE beads as described by the manufacturer (Pierce Chemical Co.). For immunoblotting the nitrocellulose sheets were blocked in 10% non-fat milk in TTBS for 4 h and incubated overnight in a 1:100 dilution of the affinity-purified antibody. After a 30 min incubation with a 1:5000 dilution of HRP-conjugated donkey anti-rabbit Ab (Amersham) the blots were developed using an enhanced chemiluminescence kit.

Lectin blotting and digestion with N-glycopeptidase F

Nitrocellulose sheets containing transferred proteins were blocked for 4 h in 0.5% (w/v) casein dissolved in Tris-buffered saline (TBS) and then incubated overnight with a 20 $\mu\text{g}/\text{ml}$ solution of biotinylated concanavalin A (ConA; Sigma Chemical Co.) in TBS containing 1 mM each of CaCl_2 , MgCl_2 and MnCl_2 . After 3×5 min washes in TBS, the sheets were incubated for 2 h with a 1:1600 dilution of HRP-coupled avidin D and the colour reaction was carried out as described for immunoblotting. N-Glycopeptidase F digestion was carried out on IP_3R immunoprecipitate from detergent extracts of control or trypsin-treated membranes. The Protein A–Sepharose beads obtained from immunoprecipitating 100 μg of detergent extract protein were washed once in 10 mM sodium phosphate buffer (pH 8.0) containing 1% Nonidet P-40, resuspended in 15 μl of 0.5% SDS and 0.1 M β -mercaptoethanol and boiled for 3 min. A further 30 μl of a buffer containing 2% Nonidet P-40, 20 mM sodium phosphate (pH 8.0), 20 mM EDTA, 10 $\mu\text{l}/\text{ml}$ proteinase-inhibitor cocktail and 14 units/ml N-glycopeptidase F (Genzyme Corporation, Boston, MA, U.S.A.) was added. The samples were incubated for 18 h at 37 °C, quenched with SDS sample buffer and processed on SDS/PAGE (10% gels) for lectin and immuno-blotting.

Measurement of [^3H] IP_3 binding

Binding of [^3H] IP_3 to microsomal membranes was measured routinely using labelled ligand at a concentration of 3 nM (17 Ci/mmol; New England Nuclear) and a filtration assay as described previously [28]. Binding of [^3H] IP_3 to solubilized extracts was measured using a poly(ethylene glycol) precipitation assay. Aliquots of the extract were incubated at 4 °C in a final volume of 120 μl in a buffer containing 120 mM KCl, 20 mM Tris/HCl (pH 8.1), 1 mM EDTA and 3 nM [^3H] IP_3 . After 10 min, 50 μl of 10 mg/ml γ -globulin and 0.83 ml of 15% (w/v) poly(ethylene glycol) (average molecular mass 8 kDa) were added and the incubation was continued for a further 30 min. Bound receptors were precipitated by centrifugation at 13000 g for 10 min. The pellets were solubilized overnight in 0.5 ml of 0.1 M NaOH and radioactivity was counted in Ecolume (ICN).

Affigel- IP_3 resin chromatography

The affigel- IP_3 resin was prepared as described [32] and was kindly provided by Dr. Glenn Prestwich (State University of New York, Stony Brook, NY, U.S.A.). Microsomal membranes (5–7 mg) were treated with trypsin, and membrane and soluble fractions were recovered as described above. The membrane pellet was resuspended in 0.1 M Na_2CO_3 (pH 11) and re-centrifuged at 100000 g . The supernatant was neutralized and combined with the original soluble fraction obtained after trypsin treatment. The pooled fractions were mixed with 2 ml of packed Affigel- IP_3 resin previously equilibrated in trypsin treatment buffer. After shaking for 1 h at room temperature, the beads were recovered by centrifugation and washed twice in trypsin treatment buffer. Bound fragments were eluted from the beads by a 1 h incubation with 2 ml of trypsin treatment buffer containing an additional 0.5 M KCl. The beads were centrifuged and eluted again with a further 2 ml of the same buffer containing an additional 10 μM IP_3 . The two eluates were concentrated on a Centricon-30 (Amicon Corp.), supplemented with 20 μg of γ -globulin as a carrier, and the entire fraction was precipitated with trichloroacetic acid.

RESULTS

Tryptic fragments recognized by anti-C-terminus antibody

Figure 1(a) shows that the C-terminal peptide IP₃R antibody recognized a single 240 kDa polypeptide that is entirely membrane-bound and represents the active IP₃R present in these membranes. Cleavage with increasing concentrations of trypsin produced a series of fragments associated with a decline in the amount of the 240 kDa species. After 15 min of incubation of the membranes with 1 µg/ml trypsin, five membrane-bound fragments retaining immunoreactivity were reproducibly observed. The smallest of these had a molecular mass of 94.0 ± 3.4 kDa (mean $\pm$ S.E.M. of four determinations). As the trypsin concentration was raised to 5 µg/ml, this species predominated, and at 10 µg/ml it remained the only tryptic fragment visible on the immunoblots. The 94 kDa species was formed within 1 min of incubation with 10 µg/ml trypsin, was stable for at least 5 min and was then progressively hydrolysed (Figure 1b). Smaller immunoreactive fragments derived from the hydrolysis of the 94 kDa species were not detected on gel systems that resolved polypeptides having molecular masses greater than 18 kDa (results not shown). These data indicate that the 94 kDa species represents the smallest membrane-bound tryptic fragment of the IP₃R retaining the C-terminal epitope recognized by the antibody.

Figure 1 Tryptic digestion of cerebellum microsomal membranes

Microsomal membranes were solubilized with trypsin using the conditions described in the Materials and methods section. Pellet (P) and supernatant (S) fractions were fractionated on SDS/7% PAGE and immunoblotted on nitrocellulose using C-terminal IP₃R antibody and ¹²⁵I-labelled goat anti-rabbit IgG as a secondary probe. (a) Autoradiogram of a representative experiment in which membranes (1 mg/ml) were incubated with 0, 5 and 10 µg/ml trypsin for 15 min at room temperature. (b) Autoradiogram of a representative experiment in which membranes (1 mg/ml) were treated with 10 µg/ml trypsin for 0, 1, 5 and 15 min. The arrow marks the position of the 94 kDa tryptic fragment.

Figure 2 Tryptic digestion of phosphorylated IP₃R

Cerebellum microsomes (1 mg of protein) were incubated (5 min, 30 °C) in 1 ml of a buffer containing 120 mM KCl, 20 mM Tris/HCl (pH 7.8), 0.3 mM MgCl₂, 0.1 mM sodium vanadate, 100 units of the catalytic subunit of beef heart cAMP-dependent protein kinase (Sigma Chemical Co.) and 100 µCi of [γ -³²P]ATP. The incubation was split into equal aliquots and one was treated with 5 µg of trypsin for 1 min. The reactions were quenched by addition of a 10-fold excess of soybean trypsin inhibitor and 2 mM EDTA. Membranes were isolated by centrifugation at 100 000 g, solubilized in 1% Triton X-100, and 100 µg portions of the control and trypsin-treated extracts were immunoprecipitated with IP₃R antibody. The immunoprecipitates were run on SDS/5% PAGE and transferred to nitrocellulose, which was first autoradiographed (a) and then Western-blotted with IP₃R antibody (b). The arrow indicates the 94 kDa fragment.

Figure 3 ConA binding to the tryptic fragment of IP₃R

Control and trypsin-treated cerebellum microsomes were solubilized and detergent extracts were immunoprecipitated as described in the Materials and methods section. The control and trypsin immunoprecipitates were separated by SDS/PAGE on 7% gels and transferred to nitrocellulose. These were then processed with IP₃R antibody (a) or biotinylated ConA (b). The arrowheads indicate the undigested IP₃R and the 94 kDa tryptic fragment.

Phosphorylation and glycosylation status of the 94 kDa tryptic fragment

In order to obtain additional information on the site cleaved by trypsin, we made use of the known localization of two cyclic AMP (cAMP)-dependent phosphorylation sites at serine residues 1589 and 1755 in the rat IP₃R [11]. Membranes were first phosphorylated in the presence of [γ -³²P]ATP and A-kinase, and then subjected to trypsin treatment. The control and digested membranes were detergent-solubilized and the IP₃R in the extracts was immunoprecipitated (Figure 2). In agreement with previous studies [17], the IP₃R in untreated cerebellum micro-

Figure 4 Effect of trypsin digestion on [³H]IP₃ binding

Cerebellum microsomes (1 mg) were incubated with 20 μ g of trypsin in a final volume of 1 ml. At the indicated times a 10-fold excess of soybean trypsin inhibitor was added and the samples were centrifuged at 100 000 *g* for 30 min. The pellets were resuspended in 0.2 ml of digestion buffer without trypsin and used for measurement of membrane [³H]IP₃ binding. The supernatants were concentrated to approx. 0.5 ml using a Centricon-30 filtration apparatus, and 15 μ g of protein was used to assay soluble binding. Non-specific binding was measured in the presence of 10 μ M unlabelled IP₃. The data shown are the means $\pm$ S.D. of triplicate determinations from a representative experiment.

somes was readily phosphorylated by A-kinase. However, treatment with trypsin generated a 94 kDa fragment that did not retain radio activity and must therefore be missing the phosphorylation site. It is known that serine-1755 is preferentially phosphorylated by A-kinase [19]. Our data would suggest that stepwise proteolysis eventually cleaves the receptor somewhere between serine-1755 and the first transmembrane loop to generate the 94 kDa fragment.

The IP₃R purified from rat cerebellum is known to be glycosylated, based on the observations that the protein adheres to ConA–Sephadex columns [2]. In order to investigate whether the 94 kDa tryptic fragment retains the sugars present in the native receptor, Western blots containing the immuno-

precipitated 94 kDa fragment were probed with biotinylated ConA. The 94 kDa tryptic fragment retained reactivity to biotinylated ConA (Figure 3b). Additional experiments (results not shown) indicated that the ConA reactivity of the immuno-precipitated 94 kDa fragment was removed by digestion with *N*-glycopeptidase F and was associated with only a small decrease in the molecular mass of the tryptic fragment (< 2 kDa).

Release of soluble [³H]IP₃ binding sites by trypsin

Figure 4 shows the measurements of [³H]IP₃ binding to membrane and soluble proteins prepared from microsomes treated for increasing periods of time with 20 μ g/ml trypsin. A progressive loss of membrane binding sites was observed, with complete loss of binding by 60 min. This loss of membrane binding sites was accompanied by a corresponding increase of binding sites in the soluble fraction. Quantitatively, the total amount of soluble binding sites detected after trypsin treatment was consistently greater than that lost from the membranes. The reason for this is not clear but may be related to differences in the membrane and soluble binding assays or to differences in the IP₃ affinity of membrane and soluble binding sites. The disappearance of membrane binding sites was not simply a reflection of a decrease in binding affinity, because loss of membrane binding sites and appearance of soluble binding sites were also observed when a near-saturating concentration of [³H]IP₃ (125 nM) was used in the binding assay (results not shown).

Mutational analysis of the IP₃R has already established that the ligand binding domain is located within the N-terminal 650 amino acids present in the large cytoplasmic portion of the receptor [14,15]. Our data would indicate that this portion is released from the microsomal membrane upon limited proteolysis in a form that retains ligand binding. The 94 kDa species would represent the portion of the receptor protein, missing the ligand binding domain, but retaining transmembrane loops and associated C-terminus. In order to recognize soluble putative ligand binding receptor fragments, we utilized a second antibody (KEEK-Ab) raised to amino acids 401–414 of the rat type-I IP₃R sequence. Figure 5(a) shows the result of immunoblotting soluble and pellet fractions prepared from the membranes at various times after trypsin treatment. KEEK-Ab recognized a 68 kDa polypeptide (67.6 ± 1.3 kDa; *n* = 4) released by trypsin treatment into the soluble fraction (Figure 5a). Several trypsin-generated polypeptides retaining the KEEK epitope, including the 68 kDa

Figure 5 Recognition of tryptic fragments by KEEK-Ab

The soluble (a) and pellet (b) fractions were obtained from the same experiment as shown in Figure 4. The soluble fractions were precipitated with 10% trichloroacetic acid. Both sets of samples were run on SDS/10%-PAGE, transferred to nitrocellulose and immunoblotted with a polyclonal antibody to amino acids 401–414 of the type-I IP₃R (KEEK-Ab). Approximately 20 μ g of protein was loaded in each lane.

Figure 6 Extraction of KEEK-reactive fragments from membranes with Na_2CO_3

Cerebellum microsomal membranes (0.5 mg of protein) were treated with 10 $\mu\text{g}/\text{ml}$ trypsin for 5 min and centrifuged at 100 000 g . The protein in the supernatants was precipitated with trichloroacetic acid as in Figure 5 (lanes 2 and 5). The trypsin-treated membranes were reisolated by centrifugation and resuspended in (a) 0.5 ml of buffer or (b) 0.5 ml of Na_2CO_3 (pH 11.0). The membranes were again centrifuged and pellets (lanes 1 and 4) and supernatant fractions (lanes 3 and 6) were processed by SDS/PAGE and immunoblotting with KEEK-Ab.

band, were also found associated with the pellet fraction (Figure 5b). This figure also shows the generation of a characteristic set of larger immunoreactive polypeptides that represent intermediate digestion products. None of these polypeptides were immunoreactive when KEEK-Ab was preincubated with the peptide corresponding to amino acids 401–414 (results not shown). In some experiments the 68 kDa band appeared as a doublet of a major and a minor band (cf. Figure 7), and there was also experimental variation in the exact time course of the appearance of soluble binding sites. However, the appearance of soluble ligand binding sites and soluble immunoreactive 68 kDa fragment were temporally correlated when both parameters were measured in the same experiment (e.g. Figures 4 and 5a).

Interaction of the N-terminal segment with the C-terminus

Figure 5(b) shows that a significant proportion of the 68 kDa KEEK-immunoreactive polypeptide remains associated with the membrane after trypsin digestion. The nature of the interaction of this N-terminal fragment with the membrane has been further investigated in the experiments shown in Figures 6 and 7. Treatment of microsomal vesicles with 0.1 M Na_2CO_3 (pH 11.0) has been established to release peripheral and intraluminal proteins, whereas integral membrane proteins remain in the pellet fraction [33]. Incubation of trypsin-treated membranes with Na_2CO_3 led to the complete removal of the 68 kDa fragment from the membrane (Figure 6, lanes 1 versus 4) and appearance of the 68 kDa polypeptide in the Na_2CO_3 supernatant (Figure 6, lanes 3 versus 6). The 94 kDa tryptic fragment, recognized by the C-terminal antibody, remained in the pellet fraction after Na_2CO_3 treatment (results not shown and Figure 7, lane 3). These data suggest that the N-terminal 68 kDa fragment is peripherally associated with the membrane. The experimental data in Figures 4 and 5 show that the membranes rapidly lose binding activity

Figure 7 The C-terminal antibody immunoprecipitates the 68 kDa KEEK-reactive fragment

Cerebellum microsomal membranes (0.5 mg of protein) were incubated in the absence (lanes 1 and 4) or presence (lanes 2, 3, 5 and 6) of 10 $\mu\text{g}/\text{ml}$ trypsin for 5 min and centrifuged at 100 000 g . The membranes were resuspended in 0.5 ml of solubilization buffer containing 1% Triton X-100. Detergent extracts were immunoprecipitated with C-terminal antibody as described in the Materials and methods section. One set of membranes was treated with Na_2CO_3 as described in Figure 6 prior to solubilization and immunoprecipitation (lanes 3 and 6). Equal aliquots of the washed immunoprecipitates were run on SDS/10% PAGE, transferred to nitrocellulose and immunoblotted with either C-terminal antibody (lanes 1–3) or KEEK-Ab (lanes 4–7). Lane 7 contains the trichloroacetic acid-precipitated protein obtained from the supernatant of Na_2CO_3 -treated trypsinized membranes.

after limited proteolysis and yet contain bound 68 kDa fragment. The fraction released by Na_2CO_3 washing of trypsin-treated membranes contains ligand binding activity (results not shown). This suggests that the conformation of the membrane-attached 68 kDa fragment generated after trypsin addition is different from that of the soluble 68 kDa fragment, and that the membrane-attached form has low ligand binding activity.

The peripheral membrane associated of the 68 kDa fragment does not appear to involve disulphide bonds, because the trypsin digestion medium contained 1 mM DTT and the migration of the immunoreactive fragments in the membranes was not different when analysed on non-reducing SDS/PAGE (results not shown). We tested the hypothesis that the interaction of the N-terminal 68 kDa fragment with the membrane may involve an association with the C-terminal portion of the receptor. This was done by using the anti-C-terminus antibody to immunoprecipitate the receptor fragments from Triton X-100 extracts of undigested and digested membranes (Figure 7). The immunoprecipitates were immunoblotted on both C-terminal and KEEK antibodies. As shown previously, the C-terminal antibody recognized a predominant band of 94 kDa generated by trypsin treatment (Figure 7, lane 2). However, the C-terminal antibody also immunoprecipitated a 68 kDa band recognized by the KEEK antibody and having the same mobility as the KEEK immunoreactive fragment found in Na_2CO_3 washes of trypsin-treated membranes (Figure 7, lane 5 and 7). Pretreatment of the membranes with Na_2CO_3 after trypsin digestion did not alter the immunoprecipitation of the 94 kDa fragment, but completely prevented the immunoprecipitation of the 68 kDa KEEK-reactive fragment (lanes 3 and 6). The converse experiment, in which KEEK-Ab is used to immunoprecipitate the C-terminal fragment, was not possible, as KEEK-Ab failed to immunoprecipitate the intact IP_3R from Triton X-100 extracts.

Figure 8 Binding of soluble immunoreactive receptor fragment of Affigel-IP₃ resin

Fractions containing trypsin-solubilized receptor fragments were prepared and incubated with Affigel-IP₃ resin as described in the Materials and methods section. Fractions were precipitated with trichloroacetic acid and immunoblotted with KEEK-Ab. Lanes 1 and 2 contain the trichloroacetic acid precipitates equivalent to 0.2 ml of extract before and after 1 h of incubation with the Affigel resin. Lane 3 corresponds to the trichloroacetic acid precipitate of the eluate obtained with 0.5 M KCl, and lane 4 is the eluate obtained with 0.5 M KCl and 10 μ M IP₃.

Binding of the 68 kDa fragment of Affigel-IP₃ resin

To obtain additional evidence for the identity of the soluble ligand binding domain, we made use of an affinity matrix containing IP₃ attached to an Affigel resin that has previously been utilized for the purification of the cerebellar IP₃R [32]. The soluble fraction obtained after trypsin treatment was pooled with the Na₂CO₃ wash of trypsin-treated membranes and incubated with the Affigel-IP₃ resin. The beads were washed and then eluted using 0.5 M KCl alone or in combination with 10 μ M IP₃. Figure 8 shows an immunoblot analysis with KEEK-Ab of fractions obtained before and after incubation of the trypsin-solubilized fractions with Affigel-IP₃ resin. The data show that the immunoreactive 68 kDa fragment was depleted as a result of a 1 h incubation with Affigel-IP₃ resin (Figure 8, lanes 1 and 2). Some of the bound 68 kDa fragment was eluted from the beads by treatment with high salt concentrations (lane 3). A more effective elution of the 68 kDa fragment was obtained with a combination of high salt and IP₃. These data are consistent with the presence of an IP₃ binding site on the 68 kDa fragment.

DISCUSSION

Our data show that limited digestion of the IP₃R in rat cerebellum membranes generates a 94 kDa integral membrane fragment which retains the C-terminal epitope and ConA reactivity, but is missing the A-kinase phosphorylation sites. The carbohydrate present on the 94 kDa fragment is N-linked to the polypeptide as shown by its removal after treatment with *N*-glycopeptidase F. Covalent attachment of sugars to susceptible asparagine residues is believed to occur only in the endoplasmic reticulum lumen [34]. There are 21 consensus N-glycosylation sites present in the type-I IP₃R sequence [10,11], of which 10 sites are present between the A-kinase phosphorylation site at serine-1755 and the C-terminus. Folding of the protein according to the eight-transmembrane-loop model originally proposed for the IP₃R on the basis of hydropathy analysis [11,14,22] would put all the glycosylation sites facing the cytosol. This is clearly incompatible with the experimental data. More recently, a revised hydropathy analysis

applied to the sequences of the IP₃R cloned from *Xenopus* [13] and *Drosophila* [12] cDNAs has led to a six-transmembrane-loop model [26]. Our data would be consistent with this revised model, which places two N-glycosylation sites between putative loops 5 and 6 within the endoplasmic reticulum lumen. Site-directed mutagenesis experiments suggesting that both sites in the mouse type-I IP₃R are glycosylated have been reported recently [27].

Limited digestion of cerebellar membranes with trypsin released [³H]IP₃ binding sites into the soluble fraction (Figure 4). In order to follow the release of some of the soluble N-terminal fragments, we raised an antibody to the peptide sequence corresponding to amino acids 401–414 of the type-I IP₃R. This KEEK-Ab did not recognize the native protein and was incapable of immunoprecipitating the receptor from detergent extracts of cerebellum microsomal membranes (results not shown). However, the antibody recognized intact and proteolytic receptor fragments on Western blots. This suggests that in the native receptor the peptide epitope may be hidden by protein folding and association with other parts of the receptor. The KEEK-Ab recognized a 68 kDa tryptic fragment released into the soluble fraction. Three lines of evidence suggest that the immunoreactive 68 kDa fragment encompasses the ligand binding site of the receptor. First, the time course of the appearance of the 68 kDa fragment correlated with the time course of the appearance of soluble [³H]IP₃ binding sites. Secondly, the 68 kDa fragment bound to immobilized IP₃ and could be eluted from the affinity matrix by added IP₃. Thirdly, the 68 kDa fragment was recognized on immunoblots by a polyclonal antibody raised to amino acids 467–484 (results not shown). This stretch of sequence within the receptor has previously been shown to be involved in binding to photolabelled IP₃ [16]. Our data are consistent with previous studies showing that the ligand binding site can be independently expressed as soluble recombinant proteins encoding the N-terminal 736 [15] or 788 [35] amino acids of the receptor.

We have found that the major portion of the immunoreactive fragments formed from the receptor after limited trypsin treatment is not released into the soluble fraction but remains tightly associated with the membranes. Attachment of the KEEK-immunoreactive fragments was disrupted by treatment of the membranes with Na₂CO₃, whereas a detergent was required for solubilization of the 94 kDa C-terminal trypsin fragment. The 68 kDa KEEK-immunoreactive fragment was specifically immunoprecipitated by antibody directed at the receptor's C-terminus, suggesting that the C-terminal transmembrane domain and a segment of the N-terminal domain are held together by non-covalent interactions. This does not necessarily require that the two domains are in direct contact, since it is also feasible that both domains interact non-covalently with a third interposed fragment which is not recognized by either of the two IP₃R antibodies. In a recent study mapping the tryptic fragments formed from digestion of the ryanodine receptor in sarcoplasmic reticulum vesicles, it was also noted that none of the immunoreactive fragments were released into the soluble fraction [36]. Some of the fragments derived from the N-terminal region could be released by Na₂CO₃ treatment. Both the peripherally associated and transmembrane segments were found to co-migrate at the bottom of sucrose-density gradients. On this basis, it was suggested that the association of N-terminal and C-terminal fragments reflects the involvement of both regions in oligomerization of the ryanodine receptor. Since oligomerization of the IP₃R has been proposed to involve only the transmembrane domain [35], it was predicted that the type of domain interactions observed in the ryanodine receptor would not be observed for the IP₃R. However, the behaviour of the IP₃R appears similar to that

of the ryanodine receptor, suggesting that the interactions between widely separated domains in both of these Ca^{2+} channels may serve some function other than oligomerization of subunits.

The functional significance of interactions between receptor domains is presently not clear, but three possibilities should be considered. First, folding of the receptor may bring the ligand binding and channel domains into close proximity, and this would clearly have important implications for how the channel is gated by IP_3 . A monoclonal antibody (18A10) recognizing an epitope at the C-terminus of IP_3R has been shown to competitively inhibit IP_3 -mediated Ca^{2+} release and to stimulate IP_3 binding [37]. This effect of 18A10 on ligand binding has been viewed as the result of a conformational change in the protein occurring upon antibody binding to the C-terminus. The possibility that the N-terminal ligand binding domain may be in close proximity to the 18A10 epitope lends further support to this explanation.

Another possibility is that the association between N- and C-terminal domains has some structural significance. It has been reported that stacks of endoplasmic reticulum cisternae seen in cerebellar Purkinje cells or COS cells overexpressing IP_3R contain crystalline arrays of IP_3R [38,39]. The gaps between adjacent stacked membranes contain bridge structures thought to be made of 'head-to-head' contacts between the N-terminal receptor domains. However, 'head-to-tail' interactions between receptors bridging the cisternal space, or lateral interactions between adjacent receptors, may also play a role in maintaining the structure of endoplasmic reticulum stacks. Finally, it cannot be excluded that the peripheral association of N-terminal tryptic fragments with the membrane reflects non-specific interactions occurring after the initiation of proteolysis. However, this seems less likely in view of the finding that the 68 kDa KEEK-immunoreactive fragment is specifically immunoprecipitated by antibody directed to the receptor's C-terminus. Further work with cross-linking agents should help to address this issue.

The cytoplasmic domain of the IP_3R expressed in COS cells has been shown to undergo a large conformational change upon binding IP_3 [35]. This change corresponded to an apparent decrease of 50 kDa in molecular mass as measured by mobility in a gel filtration column. These data have led to a model in which the ligand binding domain is viewed as being widely separated from the channel-forming transmembrane domain at the C-terminus, with the large ligand-induced conformational change being required for channel gating [35]. If such large conformational changes also occur in the full-length receptor in its native membrane environment, it would be anticipated that IP_3 would alter the surface accessibility of one or more proteinase-accessible sites. Using trypsin, chymotrypsin, endoprotease glu-C or proteinase-K and either the C-terminal antibody or KEEK-Ab as probes, we have been unable to detect any change in cleavage pattern when cerebellum microsomes are preincubated with IP_3 (results not shown). This would suggest that any conformational changes mediated by IP_3 in the native protein may be considerably more subtle than those observed in truncated soluble receptor constructs. Our data would also suggest that the current IP_3R spatial domain model, which does not envisage any intramolecular non-covalent interactions between widely separate domains, requires re-evaluation.

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