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Structure of a novel $InsP_3$ receptor

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Inositol 1,4,5-trisphosphate ($InsP_3$) constitutes a major intracellular second messenger that transduces many growth factor and neurotransmitter signals. $InsP_3$ causes the release of Ca^{2+} from intracellular stores by binding to specific receptors that are coupled to Ca^{2+} channels. One such receptor from cerebellum has previously been extensively characterized. We have now determined the full structure of a second, novel $InsP_3$ receptor which we refer to as type 2 $InsP_3$ receptor as opposed to the cerebellar type 1 $InsP_3$ receptor. The type 2 $InsP_3$ receptor has the same general structural design as the cerebellar type 1 $InsP_3$ receptor with which it shares 69% sequence identity. Expression of the amino-terminal 1078 amino acids of the type 2 receptor demonstrates high affinity binding of $InsP_3$ to the type 2 receptor with a similar specificity but higher affinity than observed for the type 1 receptor. These results demonstrate the presence of several types of $InsP_3$ receptor in brain and raise the possibility that intracellular Ca^{2+} signaling may involve multiple pathways with different regulatory properties dependent on different $InsP_3$ receptors.

Key words: Ca^{2+} channel/endoplasmic reticulum/intracellular Ca^{2+}/ryanodine receptor/signal transduction

Introduction

The cellular responses to many growth factors and neurotransmitters is mediated by increases in intracellular Ca^{2+} caused by the release of $InsP_3$ (Berridge and Irvine, 1989). $InsP_3$ binds to specific intracellular receptors and causes the release of Ca^{2+} from intracellular Ca^{2+} stores that are probably part of the endoplasmic reticulum (Streb *et al.*, 1983). Generation of intracellular $InsP_3$ also leads to the delayed influx of Ca^{2+} via plasma membrane channels by an unidentified mechanism (Penner *et al.*, 1988). In addition, $InsP_3$ is instrumental in establishing or maintaining Ca^{2+} oscillations in many cells (Woods *et al.*, 1986; Berridge, 1990; Harootunian *et al.*, 1991; Petersen *et al.*, 1991).

An $InsP_3$ receptor from cerebellum has been well characterized (reviewed in Ross *et al.*, 1990; Shears, 1991) and immunolocalized to all parts of the endoplasmic reticulum in Purkinje cells (Mignery *et al.*, 1989; Ross *et al.*, 1989). The cerebellar $InsP_3$ receptor consists of a homotetramer of M_r 313 000 subunits that are encoded by a 10 kb mRNA which is subject to at least two different alternative splicing events (Mignery *et al.*, 1989, 1990; Furiuchi et al., 1989; Ferris *et al.*, 1991a). The primary structure of the $InsP_3$ receptor predicts the presence of eight transmembrane regions (Mignery *et al.*, 1990; De Camilli *et al.*, 1990) although an alternative model with nine transmembrane regions has also been suggested (Furiuchi *et al.*, 1989). Mutagenesis studies demonstrated that the receptor forms homotetramers by virtue of intersubunit interactions localized to the regions of the membrane spanning sequences, and that the ligand binding site is localized to the amino-terminal fourth of the receptor (Mignery and Südhof, 1990). Based on these results, a domain model of the receptor was suggested whereby the receptor contains an amino-terminal binding domain, a carboxy-terminal Ca^{2+} channel domain, and an intervening coupling domain that regulates the relationship between $InsP_3$ binding and Ca^{2+} channel gating. Consistent with this model, the phosphorylation sites of the $InsP_3$ receptor were localized to the putative coupling domain (Mignery *et al.*, 1990; Ferris *et al.*, 1991b).

Although the cerebellar $InsP_3$ receptor is expressed at low levels in virtually all tissues investigated (Mignery *et al.*, 1990), several lines of evidence suggest that there may be more than one type of $InsP_3$ receptor. Biochemical data demonstrated that $InsP_3$ binding has different characteristics in different tissues and that Ca^{2+} release by $InsP_3$ may be subject to different regulatory processes in different tissues (Guillemette *et al.*, 1988; Palmer and Wakelam, 1989; Rossier *et al.*, 1989; Ely *et al.*, 1990; Pietri *et al.*, 1990). These observations raise the possibility that different tissues may express different $InsP_3$ receptors. Furthermore, it has been suggested that the endoplasmic reticulum is subcompartmentalized with respect to its function as a Ca^{2+}-storing organelle (Villa *et al.*, 1991; Takei *et al.*, 1992), indicating that different types of $InsP_3$ receptors could be expressed in different subcompartments. For example, it is conceivable that a novel type of $InsP_3$ receptor may be localized to peripheral elements of the endoplasmic reticulum and physically coupled to components of the plasma membrane, thereby mediating the observed plasma membrane Ca^{2+} flux (Irvine, 1990). Another possibility is that different $InsP_3$ receptors are localized in different compartments of the endoplasmic reticulum, conferring different Ca^{2+} release properties on these compartments.

All of these possibilities imply the presence of additional types of $InsP_3$ receptors that are distinct from the only currently described $InsP_3$ receptor. These receptors may nevertheless be structurally similar to this receptor although they differ from it in their intracellular targeting and/or

regulation. We now report the presence and full length structure of a novel type of $InsP_3$ receptor that fits these requirements. The presence of different types of $InsP_3$ receptors suggests that the intracellular Ca^{2+} signalling induced by $InsP_3$ may also be a function of the types and distributions of the $InsP_3$ receptors.

Results

In order to search for $InsP_3$ receptor related messages, a rat brain cDNA library was screened with an oligonucleotide corresponding to the last transmembrane region of the $InsP_3$ receptor (Mignery *et al.*, 1990). This region was chosen for screening because it constitutes the region of highest homology between the $InsP_3$ and ryanodine receptors (Furiuchi et al., 1989; Mignery *et al.*, 1989; Takeshima *et al.*, 1989). In addition to multiple clones encoding the cerebellar $InsP_3$ receptor, two overlapping clones were isolated that were different from the cerebellar $InsP_3$ receptor clones (pI6 and pI15, Figure 1). Sequencing demonstrated that these clones encoded a novel transcript homologous to the $InsP_3$ receptor. Oligonucleotides corresponding to the 5′ sequences of these and subsequent clones were then used to isolate further overlapping cDNA clones covering the entire coding region of the transcript and extending over 10.7 kb (Figure 1), and the sequences of all of these clones were determined.

The complete sequence of the $InsP_3$ receptor related transcript was assembled from the sequences of the overlapping cDNA clones (Figure 2). Its translated amino acid sequence predicts synthesis of a protein containing 2701 amino acids with a total molecular weight of 307 088 Daltons. The suggested initiation codon conforms well to the consensus sequence for initiator methionine codons (Kozak, 1989) and is preceded by an in-frame stop codon, suggesting that the sequence is full length with respect to the coding region. Clones containing poly(A) tails at two different positions in the 3′ untranslated region were isolated (Figure 1). Both poly(A) tails are preceded by AT-rich sequences that may serve as polyadenylation signals (underlined in Figure 2). Northern blots demonstrated the presence of two messages for this cDNA corresponding to approximately 9 and 11 kb in size (data not shown), suggesting that there is differential polyadenylation of the 3′ end of the message *in vivo*.

The amino acid sequence of the new protein was compared with that of the rat cerebellar $InsP_3$ receptor, revealing a high degree of homology between the two proteins with an overall sequence identity of 69%. Alignment of the two sequences with each other (Figure 3) demonstrated that their homology extends over their entire length but shows a patchy distribution, with regions of identity separated by completely dissimilar sequence stretches. For example, hydrophobicity plots of both sequences suggested the presence of eight transmembrane regions (Mignery *et al.*, 1990a and data not shown) which are underlined in Figure 3 and labeled M1 to M8. Most of the putative transmembrane regions are highly conserved but two transmembrane regions, M2 and M3, show very little sequence similarity. In addition, many but not all loops connecting transmembrane regions are poorly conserved, for example the sequence separating the sixth and seventh transmembrane regions contains no similarity except for two conserved cysteine residues whereas

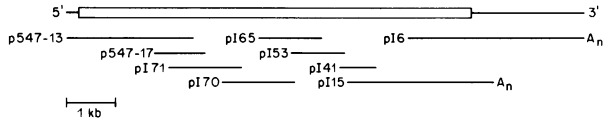

Fig. 1. Structure of the mRNA of the type 2 $InsP_3$ receptor (**top**) and distribution of the isolated cDNA clones (**bottom**). The open bar indicates the localization of the coding region in the mRNA. The scale of the graph is depicted in the lower left corner.

the transmembrane regions themselves are more than 90% identical. Similar patches of identical sequences separated by completely dissimilar regions can also be observed in other parts of the structures. In addition, deletions of one sequence relative to the other are observed, particularly in the coupling domain of the $InsP_3$ receptor that separates the transmembrane regions from the ligand binding domain. Interestingly, one of these deletions corresponds to an alternatively spliced region in the cerebellar $InsP_3$ receptor (Ferris *et al.*, 1991a), suggesting that the novel receptor may also be alternatively spliced.

Figure 3 also contains the partial sequence of a third mRNA that is related to the $InsP_3$ receptor and was isolated by the polymerase chain reaction from a human kidney cDNA library (C.L.Newton, G.A.Mignery and T.C.Südhof, in preparation). This sequence shows the same pattern of similarity and diversity as described above, suggesting that there is a family of related sequences with a similar core of conserved residues.

The strong similarity between the novel sequence described here and the cerebellar $InsP_3$ receptor suggests that the new protein may represent a new type of $InsP_3$ receptor. To test this hypothesis, we took advantage of the fact that we had previously localized the ligand binding domain of the cerebellar $InsP_3$ receptor to the amino-terminal fourth of the receptor (Mignery and Südhof, 1990). Assuming that the ligand binding site of the putative new receptor would have a similar localization, we expressed the first 1078 residues of the new sequence as a soluble protein by transient transfection in COS cells (Figure 4). The ligand binding properties of the amino-terminal fragment of the novel receptor were compared to those of the corresponding homologous fragment from the previously characterized cerebellar $InsP_3$ receptor. In order to allow recognition of the two different recombinant proteins from the two receptors, the carboxy-termini of both proteins were fused to a 12 residue peptide epitope from the carboxy-terminus of the 116 K subunit of the vacuolar proton pump (Mignery and Südhof, 1990; Perin *et al.*, 1991). After transient transfection, both proteins were expressed at high levels in soluble form in COS cells (Figure 4). Although the calculated molecular weights of the two proteins are very similar, their apparent mobility on SDS-gels differed slightly, possibly reflecting differences in their tertiary structure.

The $InsP_3$ binding properties of the recombinant proteins were then investigated in the cytosols of COS cells transfected with the expression constructs or control DNAs. Both recombinant proteins bound $InsP_3$ specifically, with the recombinant protein from the novel receptor having a slightly higher affinity than that of the corresponding fragment of the cerebellar $InsP_3$ receptor (Figure 5; apparent K_ds were 27 nM and 89.5 nM, respectively). Furthermore, in spite of the considerable sequence

differences between the two receptors, $InsP_3$ binding to the recombinant proteins was displaced by different inositol phosphates to similar extents (Table I). In addition, $InsP_3$ binding was very sensitive to heparin in both proteins. These results demonstrate that the protein described here represents a novel $InsP_3$ receptor, from now on referred to as type 2 $InsP_3$ receptor as opposed to the cerebellar type 1 $InsP_3$ receptor. In spite of their sequence differences, both receptors have similar binding specificities although different affinities.

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TCACGAGCGTGAAGCAGCATGTCTGACAAAATGTCCAGCTTCCTCTACATTGGGGACATCGTGTCCCTGTACGCGGAGGGCTCGGTCAATGGCTTCATCAGCACCCTGGGGTTG   342
                  M  S  D  K  M  S  S  F  L  Y  I  G  D  I  V  S  L  Y  A  E  G  S  V  N  G  F  I  S  T  L  G  L    32
GTGGATGACCGTTGTGTGGTGCACCCGGAGGCGGGGGATCTTACCAACCCACCCAAGAAGTTCAGAGACTGCCTCTTCAAGGTGTGCCCCATGAATCGATACTCTGCCCAGAAG   456
 V  D  D  R  C  V  V  H  P  E  A  G  D  L  T  N  P  P  K  K  F  R  D  C  L  F  K  V  C  P  M  N  R  Y  S  A  Q  K    70
CAGTACTGGAAAGCCAAGCAGGCGAAGCAGGGAAACCACACGGAGGCTGCCCTGCTGAAGAAGCTCCAGCACGCCGCAGAGCTGGAACAAAAACAGAATGAGTCAGAGAACAGG   570
 Q  Y  W  K  A  K  Q  A  K  Q  G  N  H  T  E  A  A  L  L  K  K  L  Q  H  A  A  E  L  E  Q  K  Q  N  E  S  E  N  R   108
AAACTTTTGGGAGAAATTGTGAAATACAGCAAAGTTATACAACTACTGCACATAAAAAGCAACAAGTACCTCACCGTGAACAAGAGGTTACCTGCCTTACTGGAGAAGAATGCC   684
 K  L  L  G  E  I  V  K  Y  S  K  V  I  Q  L  L  H  I  K  S  N  K  Y  L  T  V  N  K  R  L  P  A  L  L  E  K  N  A   146
ATGCGTGTGTCCCTGGATGCTGCAGGGAACGAAGGGTCCTGGTTCTACATCCATCCCTTCTGGAAGCTGAGAAGCGAGGGTGATAATATCGTCGTGGGAGATAAAGTCGTTCTG   798
 M  R  V  S  L  D  A  A  G  N  E  G  S  W  F  Y  I  H  P  F  W  K  L  R  S  E  G  D  N  I  V  V  G  D  K  V  V  L   184
ATGCCTGTAAATGCTGGGCAGCCCCTGCATGCCAGCAACGTGGAGCTCCTGGACAACCCCGGCTGCAAAGAGGTGAATGCTGTTAATTGCAACACTAGCTGGAAAATCACTTTA   912
 M  P  V  N  A  G  Q  P  L  H  A  S  N  V  E  L  L  D  N  P  G  C  K  E  V  N  A  V  N  C  N  T  S  W  K  I  T  L   222
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 Q  R  A  D  C  L  V  P  R  N  S  Y  V  R  L  R  H  L  C  T  N  T  W  V  T  S  T  S  I  P  I  D  T  E  E  E  R  P   412
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 V  L  A  T  T  V  K  K  L  E  N  G  S  I  T  Q  N  E  R  R  F  V  T  K  L  L  E  D  L  I  F  F  V  A  D  V  T  N   488
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 K  E  K  A  G  E  G  S  M  L  R  L  E  D  L  G  D  Q  R  Y  A  P  Y  K  Y  V  L  R  L  C  Y  R  V  L  R  H  S  Q   564
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 R  A  R  G  D  E  S  G  P  L  A  Y  H  I  T  L  V  E  L  L  A  A  C  T  E  G  K  N  V  Y  T  E  I  K  C  N  S  L  1400
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 L  P  L  D  D  I  V  R  V  V  T  H  D  D  C  I  P  E  V  K  I  A  Y  V  N  F  V  N  H  C  Y  V  D  T  E  V  E  M  1438
AAGGAAATCTATACAAGTAACCACATTTGGAAGTTATTTGAGAACTTCTTGGTGGATATGGCAAGGGTCTGCAACACCACCACGGACCGGAAACACGCAGACACCTTTCTGGAG  4674
 K  E  I  Y  T  S  N  H  I  W  K  L  F  E  N  F  L  V  D  M  A  R  V  C  N  T  T  D  R  K  H  A  D  T  F  L  E  1476
AGGTGTGTGACAGAGTCAGTCATGAACATCGTGAGCGGCTTCTTCAACTCGCCGTTTTCGGATAACAGTACCAGCCTCCAGACCCATCAGCCGGTCTTTATCCAGCTGCTGCAG  4788
 R  C  V  T  E  S  V  M  N  I  V  S  G  F  F  N  S  P  F  S  D  N  S  T  S  L  Q  T  H  Q  P  V  F  I  Q  L  L  Q  1514
TCCGCCTTCAGAATTTACAACTGCACCTGGCCCAACCCAGCCCAGAAAGCCTCCGTGGAATCCTGCATCAGAGCCCTGGCTGAAGTGGCCAAAAACCGTGGGATTCGCAG  4902
 S  A  F  R  I  Y  N  C  T  W  P  N  P  A  Q  K  A  S  V  E  S  C  I  R  A  L  A  E  V  A  K  N  R  G  I  A  I  P  1552
GTGGATTTGGACAGCCAGGTCAACACACTCTTCATGAAGAATCACTCAAGTACAGTGCAGAGGGCGGCCATGGGCTGGAGGCTCTCTGCTCGCTCCGGACCTCGCTTCAAGGAA  5016
 V  D  L  D  S  Q  V  N  T  L  F  M  K  N  H  S  S  T  V  Q  R  A  A  M  G  W  R  L  S  A  R  S  G  P  R  F  K  E  1590
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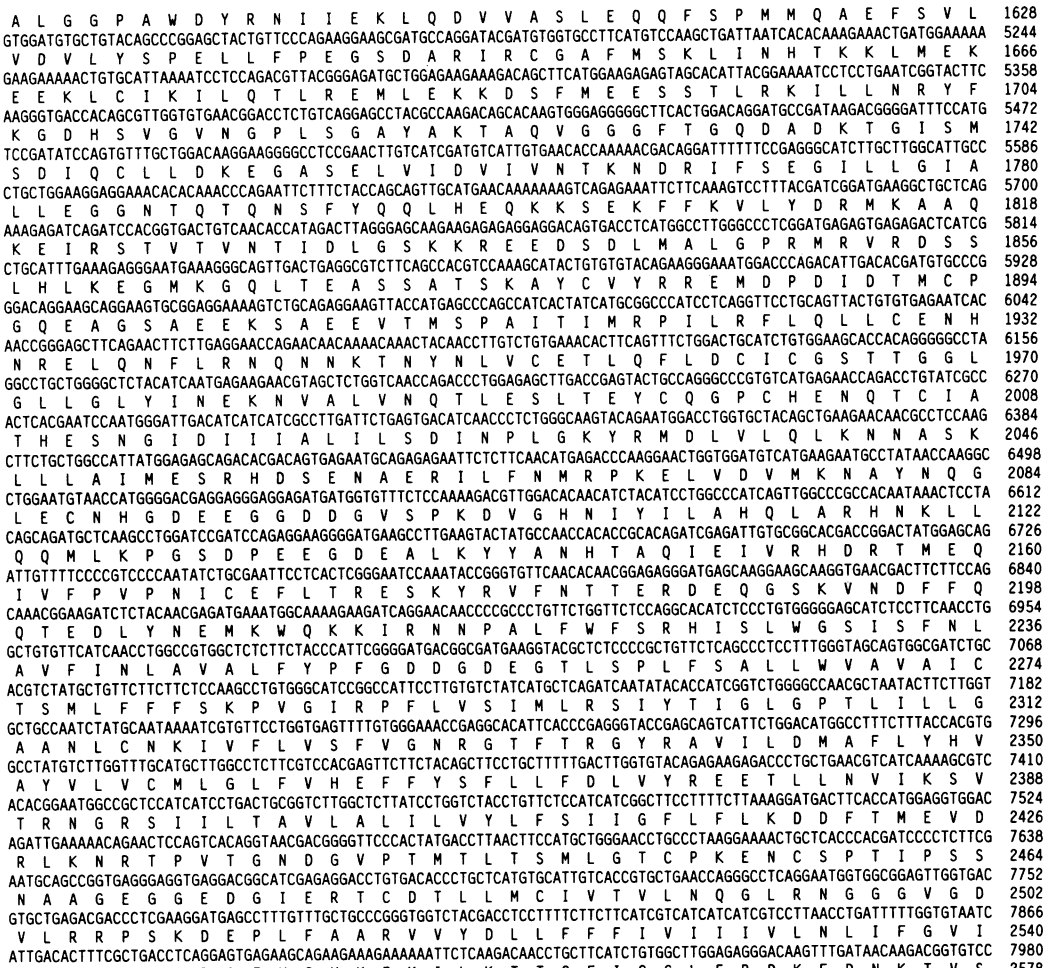

Fig. 2. Nucleotide and translated amino acid sequences of the type 2 $InsP_3$ receptor from rat. The sequence was assembled from the sequences of the cDNA clones shown in Figure 1. The deduced amino acid sequence is shown in single letter code below the nucleotide sequence, and both sequences are numbered on the right. The in-frame stop codon in the 5′ untranslated region preceding the initiator codon is underlined. The position of the poly(A) tail in pI15 is shown by an asterisk, and sequences that might serve as polyadenylation signals for this poly(A) tail and for the one at the end of clone pI6 are underlined. The following sequence differences were noted between different cDNA clones: The C at position 2289 was a T in p547-17 (silent change); the G at position 2311 was a C in pI71, changing D at position 689 to H; the G at position 2397 was a T in pI71 (silent change); the T at position 2604 was a C in p547-17 (silent change); the G at position 3283 was a T in pI70, changing G at position 1013 to C; the G at position 3473 was a C in pI70, changing G at position 1065 to A; the T at position 4013 was a C in pI70, changing L at position 1256 to P; the C at position 4770 was a T in pI65 (silent change); the G at position 4890 was an A in pI65 (silent change); the G at position 4911 was an A in pI65 (silent change); the G at position 5929 was an A in pI15 (silent change); the G at position 7396 was a A in pI15, changing V at position 2384 to I; and the A at position 8327 was a T in pI6, changing E at position 2694 to V. In addition, clone pI70 had an out-of-frame deletion from nucleotide 3311 to 3378. These sequence data are available from the EMBL/GenBank/DDBJ databases under accession number X61677 ITPR2.

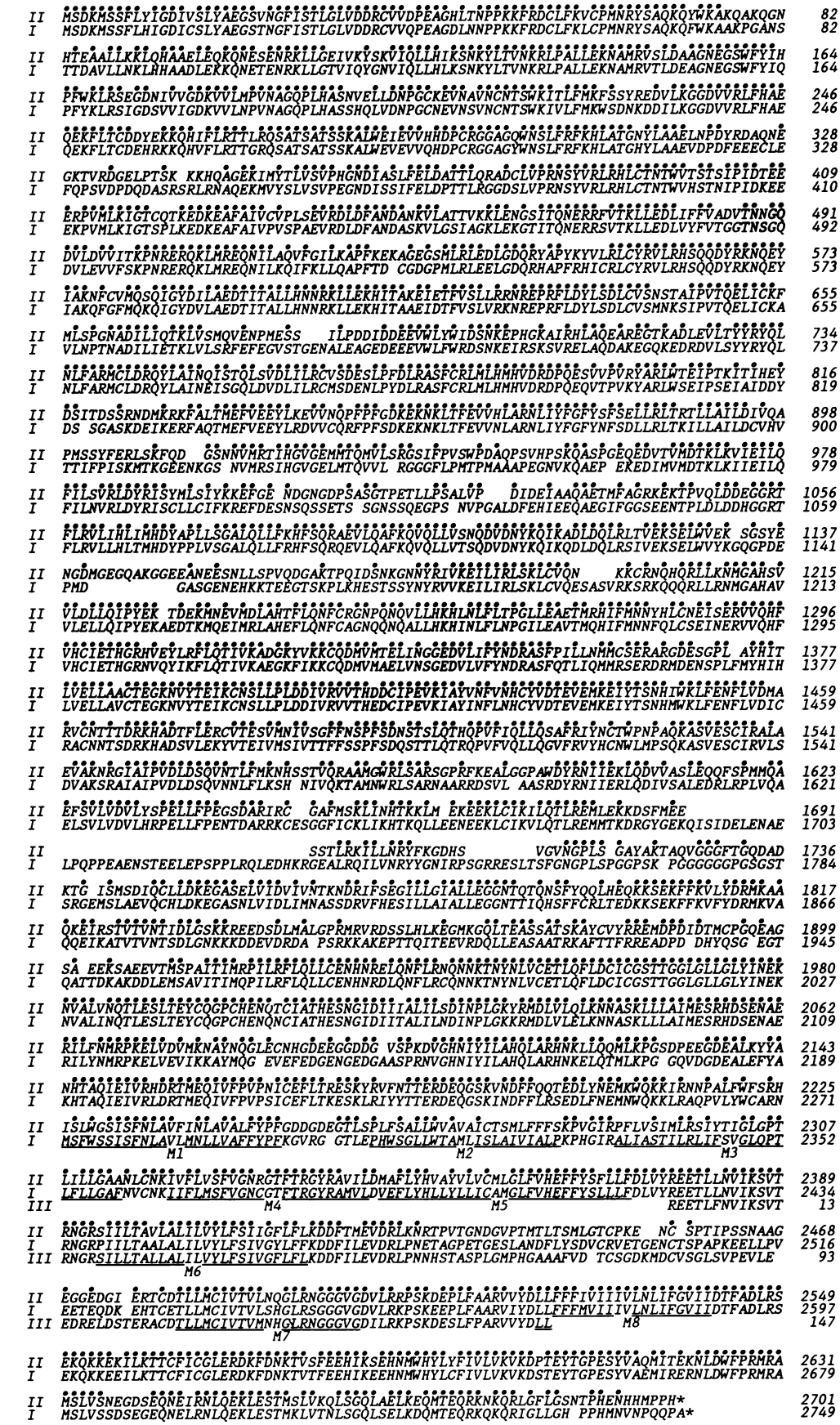

Fig. 3. Alignment of the amino acid sequence of the rat type 2 $InsP_3$ receptor (top line) with that of the type 1 receptor (second line, from Mignery *et al.*, 1990) and the partial sequence of the putative human type 3 $InsP_3$ receptor (third line; C.L.Newton, G.A.Mignery and T.C.Südhof, in preparation). Identical residues are marked by dots above the sequence. Sequences are shown in single letter code and are numbered to the right. Amino acids belonging to putative transmembrane regions are underlined and the transmembrane regions are labeled M1 to M8. The position of the stop codon is indicated by an asterisk.

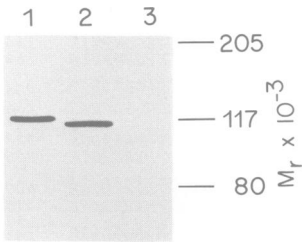

Fig. 4. Immunoblot analysis of proteins specified by the amino-terminal sequences of the type 1 and type 2 receptors expressed in COS cells. The amino-terminal 1081 and 1078 residues of the type 1 and type 2 $InsP_3$ receptors, respectively, were cloned into an expression vector fused to a sequence encoding the last 12 amino acids of the 116 K proton pump subunit. Cytosol from COS cells transfected with the type 1 $InsP_3$ receptor expression construct (pIP_3R-Stop1081, lane 1), type 2 $InsP_3$ receptor construct (pIP_3R2-Stop1078, lane 2) or with control DNA (salmon sperm DNA, lane 3) were analyzed by immunoblotting using an antibody against their common carboxy-terminal epitope followed by a peroxidase-labeled secondary antibody. Expression of the constructs results in soluble receptor proteins containing the full-length binding sites of the two $InsP_3$ receptors and ending in the same carboxy-terminal sequence.

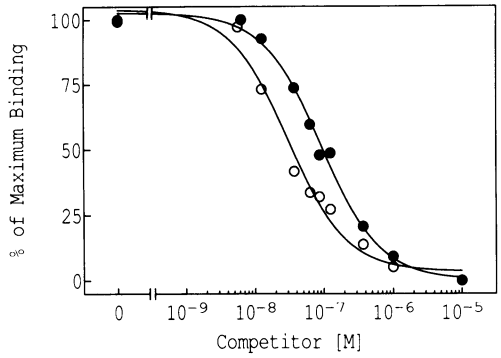

Fig. 5. Determination of the binding affinities of the ligand binding domains of the type 1 and type 2 $InsP_3$ receptors. Cytosol from COS cells transfected with constructs encoding the ligand binding domains of the type 1 (closed symbols) and type 2 $InsP_3$ receptors (open symbols) were used in binding-competition assays whereby the displacement of 3.3 nM [^{32}P]$InsP_3$ by unlabeled $InsP_3$ was determined. The line through the points represents the best fit to the data calculated using GraphPAD version 3.1 software, resulting in the determination of affinities for the type 1 and type 2 receptors of 89.5 nM and of 27.0 nM, respectively. The experiment was repeated twice with similar results. COS cells transfected with control DNA showed no measurable $InsP_3$ binding (Table I).

Table I. Binding specificities of type 1 and type 2 Ins(1,4,5)P_3 receptors

Competitor (10 μM)	pIP_3R-Stop1081 (c.p.m./mg × 10^{-3})	Percentage of control	pIP_3R2-Stop1078 (c.p.m./mg × 10^{-3})	Percentage of control	Salmon sperm DNA
–	22.86 ± 1.36	100.0	11.33 ± 0.59	100.0	0.00 ± 0.17
Inositol 1,4-bisphosphate	22.86 ± 1.17	100.0	13.61 ± 0.50	120.1	–
Inositol 4,5-bisphosphate	16.03 ± 0.47	70.1	7.71 ± 0.31	68.0	–
Inositol 1,4,5-trisphosphate	0.00 ± 0.18	0.0	0.00 ± 0.15	0.0	0.00 ± 0.96
Inositol 2,4,5-trisphosphate	0.45 ± 1.74	2.0	0.60 ± 0.48	5.3	–
Inositol 1,3,4,5-tetrakisphosphate	8.68 ± 1.20	38.0	5.05 ± 0.93	44.6	–
Inositol 1,4,5,6-tetrakisphosphate	20.13 ± 1.06	88.1	11.59 ± 0.55	102.3	–
Inositol 1,3,4,5,6-pentakisphosphate	12.17 ± 1.29	53.2	7.22 ± 0.61	63.7	–
Inositol hexakisphosphate	17.86 ± 0.49	78.1	9.46 ± 1.11	83.5	–
Heparin (5 μg/ml)	2.62 ± 0.94	11.5	3.58 ± 1.28	31.6	–
Heparin (100 μg/ml)	0.00 ± 0.50	0.0	0.00 ± 0.78	0.0	–

^{3}H-$InsP_3$-binding (25 nM) was measured with 50 μg protein of the cytosol of COS cells transfected with the indicated DNAs. Quantitative immunoblotting showed that the expression of the pIP_3R-Stop1078 was 77% of that of pIP_3R-Stop1081 (2.95 and 3.81 × 10^6 c.p.m. ^{125}I-labeled antibody binding per mg protein, respectively) with no measurable $InsP_3$ binding or receptor expression in COS cells transfected with control DNA.

Discussion

In this study we have isolated and sequenced a set of overlapping cDNA clones encoding a novel $InsP_3$ receptor. The complete primary structure of the new receptor was determined and consisted of 2701 amino acids and was found to be homologous over its entire length to the cerebellar $InsP_3$ receptor—the only $InsP_3$ receptor previously characterized. Expression of the amino-terminal domains of the novel receptor (referred to as type 2 $InsP_3$ receptor) and of the cerebellar receptor (referred to as type 1 receptor) in COS cells demonstrates that both bind $InsP_3$ with high affinity and similar specificities, although the type 2 receptor has a significantly higher affinity than the cerebellar type 1 receptor. Together our results demonstrate the presence of different types of $InsP_3$ receptors in brain tissue, whose sequences and properties suggest that they may have different $InsP_3$ binding affinities and regulatory characteristics.

Alignment of the sequences of the type 1 and type 2 $InsP_3$ receptors reveals a scattered distribution of identical and diverse sequences with an overall sequence homology of 69%. The structural design of the two $InsP_3$ receptors is similar, suggesting that they are comprised of similar functional domains. We have previously proposed a domain model for the cerebellar $InsP_3$ receptor that divides its sequence into a ligand binding domain, a coupling domain transducing the ligand binding signal, and a Ca^{2+} channel domain (Mignery and Südhof, 1990). Analysis of the sequence similarity between the receptors as a function of these domains suggests the ligand binding site is the most conserved region between the two receptors (Figure 6). This agrees well with the similar binding characteristics of the two receptors and suggests that their primary functional differences may be localized to the coupling domain and the Ca^{2+} channel domain.

The coupling domain separating the ligand binding domain from the putative channel domain is the least similar domain between the two types of receptors. The coupling domain contains the cAMP-dependent phosphorylation sites of the cerebellar $InsP_3$ receptor, suggesting that it is the principal target of regulatory signals in the $InsP_3$ receptor (Mignery *et al.*, 1990; Ferris *et al.*, 1991b). The lack of conservation between the two types of $InsP_3$ receptors in this region suggests that the two receptors may be subject to different types of regulation. In addition, significant sequence

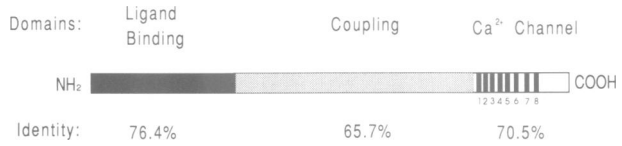

Fig. 6. Domain model of the $InsP_3$ receptors and sequence identities between the type 1 and type 2 $InsP_3$ receptors in the different domains. The three principal domains of the $InsP_3$ receptor are described on top (Mignery and Südhof, 1990). The eight putative transmembrane regions in the carboxy-terminal fourth of the receptors are numbered and their position indicated by vertical lines. The sequence identity between the two types of $InsP_3$ receptors in each domain is shown on the bottom.

differences are observed in the putative Ca^{2+} channel domain, particularly in the loops separating transmembrane regions. These differences suggest that the Ca^{2+} gating characteristics of the two receptors may also be different.

Our results demonstrate the presence of multiple types of $InsP_3$ receptors co-expressed in brain. Many of the proteins involved in signal transduction at the cell surface have been shown to be present in multiple isoforms with different regulatory properties but this is the first such demonstration for a protein functioning downstream of the generation of $InsP_3$. What is the biological relevance of the presence of different types of $InsP_3$ receptors? We would like to suggest three major hypotheses that are not mutually exclusive and are based on examples of differentially regulated isoforms of proteins involved in signal transduction cascades.

1. Different types of $InsP_3$ receptors may be functionally similar but have different regulatory properties. This would result in differences of the properties of intracellular Ca^{2+} stores dependent on which $InsP_3$ receptors are expressed. This hypothesis is supported by the fact that the putative coupling domains of the $InsP_3$ receptors that connect their ligand binding sites to the transmembrane regions is the least conserved between the receptor forms, suggesting that they may indeed be subject to differential regulation.
2. Different $InsP_3$ receptors could have different intracellular functions specified by different intracellular localizations. Currently it seems unlikely, although not excluded, that an $InsP_3$ receptor might be present in a subcellular membrane other than the endoplasmic reticulum, such as the plasma membrane (Penner *et al.*, 1988). It is more likely that there are specialized subcompartments of the endoplasmic reticulum which may contain differentially regulated Ca^{2+} stores (Lechleiter *et al.*, 1991; Villa *et al.*, 1991).
3. Different types of $InsP_3$ receptors could have different intracellular functions analogous to the two forms of the ryanodine receptor. Ryanodine receptors, similar to $InsP_3$ receptors, release Ca^{2+} from intracellular stores. The ryanodine receptors from cardiac and skeletal muscle differ from each other in the coupling between membrane depolarization to Ca^{2+} release, and their sequences are 65% identical (Takeshima *et al.*, 1989; Otsu *et al.*, 1990; Zorzato *et al.*, 1990). It is possible that of the different types of $InsP_3$ receptors, one could be autonomous in the cell interior whereas the other similar to the skeletal muscle ryanodine receptor could be coupled to the plasma membrane. The low abundance of the type 2 $InsP_3$ receptor would support such a model.

All these hypotheses (independent of which will be proved to be correct) imply that Ca^{2+} signaling induced by $InsP_3$ is much more complex than that envisioned by a single type of receptor. Clearly Ca^{2+}-release from intracellular stores is not a uniform process but dependent on receptor types as well as secondary regulatory events.

Materials and methods

cDNA cloning and DNA sequencing

A rat cDNA library was screened as described (Südhof et al., 1987; Mignery *et al.*, 1990) with an oligonucleotide complementary to the sequence of the last putative transmembrane region of both the ryanodine and the $InsP_3$ receptors (oligonucleotide sequence: CAGCTGCAGGACGATGATGAT-GACCATGAAGAAGAA). Of the 15 clones isolated, most encoded the cerebellar $InsP_3$ receptor but two clones upon sequencing were found to be distinct from but homologous to the cerebellar $InsP_3$ receptor. Although both of these clones terminated in poly(A) tails, one clone had a 1.9 kb longer 3′ untranslated region than the other, suggesting differential use of polyadenylation signals. The two clones were fully sequenced and further 5′ clones were isolated using oligonucleotides based on the sequences of the 5′ ends of these clones. The complete receptor was cloned in this manner on nine overlapping cDNA clones, several of which were isolated more than one time.

Polymerase chain reaction cloning of $InsP_3$ receptor related transcripts from a human kidney library was performed as described (Perin *et al.*, 1991) using the oligonucleotide described above as the specific primer and primers from the flanking sequences of the vector as the second primer. Only two transcripts with homology to the cerebellar $InsP_3$ receptor were isolated, one of which was the human homologue of the cerebellar receptor, whereas the second encoded a novel sequence. DNA sequencing was performed by the chain termination method (Sanger *et al.*, 1977) either manually using ^{32}P- and ^{35}S-labeled nucleotides or automatically on an ABI 370A sequencer using single-stranded M13 subclones of the cDNA clones. Sequences were analyzed on an IBM-AT computer using Microgenie software and searched against GenBank release 64 and NBRF release 25, with no significant homology observed with any sequences in the databanks except for the $InsP_3$ receptor and ryanodine receptor.

Expression of the ligand binding sites of type 1 and type 2 $InsP_3$ receptors by transfection

pIP_3R2-Stop1078 is a mammalian expression vector in which the cytomegalovirus promoter drives the expression of the first 1078 amino acids of the type-2 $InsP_3$ receptor. This sequence is followed by the 12 carboxy-terminal acids of the 116 K subunit of the proton pump (Perin *et al.*, 1991) against which we obtained an antipeptide antibody that was used both to visualize and to quantify expression. pIP_32-Stop1078 was constructed by cloning the 2.45 kb *Eco*RI–*Kpn*I fragment from p567-13 into pCMV2 (kind gift of Dr D.W.Russell, University of Texas Southwestern Medical Center, Dallas), followed by the 1.04 kb *Kpn*I–*Pst*I fragment from pI71 and by an oligonucleotide encoding the carboxy-terminal epitope. The corresponding type-1 $InsP_3$ receptor expression vector pIP_3R-Stop1081 was described previously (Mignery and Südhof, 1990). Purified DNA from both vectors was transiently transfected into COS cells and expression was analyzed by immunoblotting using peroxidase-labeled secondary antibodies and quantified using iodinated secondary antibodies and an Ambis radio-analytic imaging system. The cytosol from transfected cells was prepared as described (Mignery and Südhof, 1990) and used for binding measurements. All binding measurements were performed using the PEG precipitation assay (Chadwick *et al.*, 1990) and tritiated $InsP_3$ (17 Ci/mmole) (NEN-Du Pont) except for the assays used for the determination of the binding affinities in which ^{32}P-labeled $InsP_3$ (155 Ci/mmole) was used because of the required higher sensitivity. COS cells transfected with salmon sperm DNA were used as negative controls in all experiments. Binding data were evaluated and affinities calculated using GraphPAD InPlot version 3.1 software.

RNA blotting experiments

Total RNA was isolated from rat tissues and used for RNA-blots as described (Perin *et al.*, 1986). All blots were probed with uniformly labeled single-stranded DNA probes generated on M13 templates, and washed at high stringencies.

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