

Structure of a novel InsP₃ receptor

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Inositol 1,4,5-trisphosphate (InsP₃) constitutes a major intracellular second messenger that transduces many growth factor and neurotransmitter signals. InsP₃ causes the release of Ca²⁺ from intracellular stores by binding to specific receptors that are coupled to Ca²⁺ channels. One such receptor from cerebellum has previously been extensively characterized. We have now determined the full structure of a second, novel InsP₃ receptor which we refer to as type 2 InsP₃ receptor as opposed to the cerebellar type 1 InsP₃ receptor. The type 2 InsP₃ receptor has the same general structural design as the cerebellar type 1 InsP₃ 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 InsP₃ 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 InsP₃ receptor in brain and raise the possibility that intracellular Ca²⁺ signaling may involve multiple pathways with different regulatory properties dependent on different InsP₃ receptors.

Key words: Ca²⁺ channel/endoplasmic reticulum/
intracellular Ca²⁺/ryanodine receptor/signal transduction

Introduction

The cellular responses to many growth factors and neurotransmitters is mediated by increases in intracellular Ca²⁺ caused by the release of InsP₃ (Berridge and Irvine, 1989). InsP₃ binds to specific intracellular receptors and causes the release of Ca²⁺ from intracellular Ca²⁺ stores that are probably part of the endoplasmic reticulum (Streb *et al.*, 1983). Generation of intracellular InsP₃ also leads to the delayed influx of Ca²⁺ via plasma membrane channels by an unidentified mechanism (Penner *et al.*, 1988). In addition, InsP₃ is instrumental in establishing or maintaining Ca²⁺ oscillations in many cells (Woods *et al.*, 1986; Berridge, 1990; Harootyan *et al.*, 1991; Petersen *et al.*, 1991).

An InsP₃ 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 InsP₃ 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 InsP₃ 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 Ca²⁺ channel domain, and an intervening coupling domain that regulates the relationship between InsP₃ binding and Ca²⁺ channel gating. Consistent with this model, the phosphorylation sites of the InsP₃ receptor were localized to the putative coupling domain (Mignery *et al.*, 1990; Ferris *et al.*, 1991b).

Although the cerebellar InsP₃ 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 InsP₃ receptor. Biochemical data demonstrated that InsP₃ binding has different characteristics in different tissues and that Ca²⁺ release by InsP₃ 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 InsP₃ receptors. Furthermore, it has been suggested that the endoplasmic reticulum is subcompartmentalized with respect to its function as a Ca²⁺-storing organelle (Villa *et al.*, 1991; Takei *et al.*, 1992), indicating that different types of InsP₃ receptors could be expressed in different subcompartments. For example, it is conceivable that a novel type of InsP₃ 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 Ca²⁺ flux (Irvine, 1990). Another possibility is that different InsP₃ receptors are localized in different compartments of the endoplasmic reticulum, conferring different Ca²⁺ release properties on these compartments.

All of these possibilities imply the presence of additional types of InsP₃ receptors that are distinct from the only currently described InsP₃ 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 InsP₃ receptor that fits these requirements. The presence of different types of InsP₃ receptors suggests that the intracellular Ca²⁺ signalling induced by InsP₃ may also be a function of the types and distributions of the InsP₃ receptors.

Results

In order to search for InsP₃ receptor related messages, a rat brain cDNA library was screened with an oligonucleotide corresponding to the last transmembrane region of the InsP₃ receptor (Mignery *et al.*, 1990). This region was chosen for screening because it constitutes the region of highest homology between the InsP₃ and ryanodine receptors (Furiuchi *et al.*, 1989; Mignery *et al.*, 1989; Takeshima *et al.*, 1989). In addition to multiple clones encoding the cerebellar InsP₃ receptor, two overlapping clones were isolated that were different from the cerebellar InsP₃ receptor clones (pI6 and pI15, Figure 1). Sequencing demonstrated that these clones encoded a novel transcript homologous to the InsP₃ 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 InsP₃ 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 InsP₃ 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

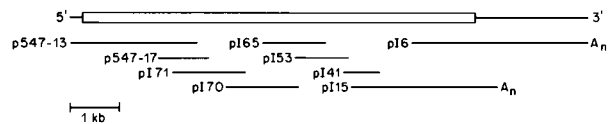


Fig. 1. Structure of the mRNA of the type 2 InsP₃ receptor (**top**) and distribution of the isolated cDNA clones (**bottom**). The open bar indicates the localization of the coding region in the mRNA. The scale of the graph is depicted in the lower left corner.

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 InsP₃ 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 InsP₃ 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 InsP₃ 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 InsP₃ receptor suggests that the new protein may represent a new type of InsP₃ receptor. To test this hypothesis, we took advantage of the fact that we had previously localized the ligand binding domain of the cerebellar InsP₃ 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 InsP₃ 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 InsP₃ 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 InsP₃ specifically, with the recombinant protein from the novel receptor having a slightly higher affinity than that of the corresponding fragment of the cerebellar InsP₃ receptor (Figure 5; apparent K_d s were 27 nM and 89.5 nM, respectively). Furthermore, in spite of the considerable sequence

differences between the two receptors, InsP₃ binding to the recombinant proteins was displaced by different inositol phosphates to similar extents (Table I). In addition, InsP₃ binding was very sensitive to heparin in both proteins. These results demonstrate that the protein described here represents

a novel InsP₃ receptor, from now on referred to as type 2 InsP₃ receptor as opposed to the cerebellar type 1 InsP₃ receptor. In spite of their sequence differences, both receptors have similar binding specificities although different affinities.

GGGACGCAGAGGGAGCGGGGACGGGAGGAGGCGAAGGTGTAGGACAGAACTTCGCCAGGAACAGGAACCCACGGCCGGCCAGGGGCGGCGGGGCGCCATC 114
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 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
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 V D D R C V V H P E A G D L T N P P K F R D C L F K V C P M N R Y S A Q K 70
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 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 A L E Q K Q N E S E N R 108
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 K L 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
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 F M K F S S Y R E D V L K G G D V V L R F H A E Q E K F L T C D D Y E K K Q 260
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 H I F L R T T L R Q S A T S A T S A L W E I E V V H H D P C R G G A G Q 298
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 W N S L F R F K H L A T G N Y L A A E L N P D Y R D A Q N E G K T V R D G E 336
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 L P T S K K K H Q A G E K I M Y T L V S V P H G N D I A S L F E L D A T T L 374
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 Q R A D L V P R N S Y V R L R L H 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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 E S V P V R Y A R L W T E I P T K I T I H E Y D S I T D S R N M K R K 830
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G L L G L Y I N E K N V A L V N Q T L E S L T E Y C Q G P C H E N Q C I A 2008
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Q Q M L K P G S D P E E G D E A L K Y A N H T A Q I E I V R H D R T M E Q 2160
ATTGTTTCCCCTGCCAATATCCGAATCTCTACTCGGAATCCAAATCCGGGTGTCAACACACAGGAGAGGATGAGCAAGGAGCAAGGTGACAGCACTTCTCCAG 6840
I V F P V P N I C E F L T R E S K Y R V N F N T T E R D E Q G S K V N D F F Q 2198
CAAAACGGAAGATCTCAACAGAGATGAAATGGCAAAAAGAGTACAGGAACAACCCGCCCTGTCTGGTCTCCAGGACATCTCCCTGTGGGGGAGCATCTCTCAACCTG 6954
Q T E D L Y N E M K W Q K K I R N N P A L F W F S R H I S L W G S I S F N L 2236
GCTGTGTCATCAACTGGCCGGTCTCTCTTCCACTCCGCGGATGACGGCAGTGAAGTACGCTCTCCCGCTGTTCAGCCCTCTTGGGATGACATCAACAGGCTCGC 7068
A V F I N L A V A L F Y P F G D G D E G T L S P L F S A L L W V A V A I C 2274
ACGTATGTCTTCTTCTTCCAGCCGTGGCATCCGGCCATCTTGTGTCTATCATGCTCAGATCAATACACCACTCGGTCTGGGGCAACGTCAATCTTGTGT 7182
T S M L F F T S K P V G I R P F L V S I M L R S I Y T I G L G P T L I L L G 2312
GCTGCCAATCTATGCAATAAATCGTGTCTCGGTGAGTTTGTGGAAACCAGGACATTCACCCAGGGTACCGAGCAGTCTTCTGGACATGGCTTTTACACAGTG 7296
A N L C N K I V F L V S F V G N R G T F T R G Y R A V I L D M A F L Y H V 2350
GCCTATGCTGTGTTGATCTGGCCCTCTCTGTCACAGGTTCTTCAAGCTTCTGCTTTTGTGATGAGTACAGAGAAAGGACCCGCTGCAAGCTCATAAAAGCTCA 7410
A Y V L V C M L G L F V H E F Y S F L L F D L V Y R E E T L L N I K S V 2388
ACACGGAATGGCCCTCATCATCTGACTGCGGTCTGGCTTATCTGGTCTACCTGTTCTCCATCATCGGCCTCCTTCTTAAAGGATGACTTACCATTGGAGGTGGAC 7524
T R N G R S I I L T A V L A L I L V L F S I I G F L F L K D D F T M E V D 2426
AGATTGAAAACAGAACTCCAGTCCAGGTAAACAGGGGTCCCACTATGACCTTAACTTCCATGCTGGAACTGCCCTAAGGAAAACGTCCACCCACGATCCCTCTTGG 7638
R L K N R T P V T G N D G V P T M T L T S M L G T C P K E N C S P T I P S S 2464
AATGACGGCTGAGGAGTGAAGGACGAGAGGACCTGTGACACCTGTCTGTCATGCTGTCACCGTGTGAACAGGGCCCTCAGGAATGGTGGGGAGTGGTGAC 7752
N A A G E G G E D G I E R T C D T L L M C I V T V L N Q G L R N G G V G D 2502
GTGCTGAGACGACCTCGAAGGATGAGCCTTGTGCTGCCGGGTGGTCTACGACCTCCTTCTTCTCATCATCATCTCACTTAACTGATTTTGGTGTAACT 7866
V L R R P S K D E P L F A A R V V D L L F F F I V I I V L N L I F G V I 2540
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I D T A F D L R S E K Q K E K I L K T T C F I C G L E R D K F 2578
TTTGAGGACACATCAAGTCAAGCACAACATGTGGCATTACTGTACTCATCTGCTGTTGGTGAAGTGAAGGACCAACAGAAATACACAGGGCCCTGAGAGACTGCTGGCTCAG 8094
F E E H I K A S E H N M W H Y L F I V L V K V K D P T E Y T G P E S Y V A Q 2616
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M I T E K N L D W F P R M R A M S L V S N E G D S E Q N E I R N L Q E K L E 2654
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S T M S L V K Q L S G Q L A E L K E Q M T E Q R K N K Q R L G F L G S N T P 2692
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H E N H H M P P H 2701
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AAAAACAACAGTATAGGGCAGACCCTCCTCATGTGGCAGGAAAGCGCCAGCTGAGGGGGTGGAGAGTCTGATCGGGGAGCGGACAGCTCCCTCCGAT 8664
CGATAGGCCCTGGACTGTCTCACACACTGACTGCAGTGTCCATCGTGGCTGGTAAATTTTTTCTCAAACTGTGGCCTGGGTCAGCGAGACAGGAAAGCCACACTCTGCT 8778
GGCTAAGTCTAAGAAATTTAAAGATTTAAAGAGGAGAAATGAAAAGGGTGTGTTAACTTCCGAACCTTACGTGTTAACTGGACATTTCTTCTTGGCATGAGAGGGGCTCAG 8892
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GTGCTGGCATGGCAAGCTGAGGGGGTTGGGGGAACTAAGAAATGCAATGACTAGCTGAAGAAATGCAACAACTTCAACAGCACATTTAAGGCTGTAGAGTAAAGCTTT 9348
TCAAATGTGAGGACATCAATCTTCCCTCCCTCACTGTTTCTGACTGATCAACAGAGTATGTTGCAAGGAAAATAAATTTGGAAGGAGAGATTCCTTTTGGAGAG 9462
TGCTGAGGTGTGAGAGACAGAGATCATGAAAGTGAAGCAGTGGTGGTGGGAGGGGACCGACAGCACTACATCTGAAACAGAGAGACTCTTCTGAGTCCGAGAT 9576
CTTAACTCTGAGCCAGGGCCACATGGGGTTGTGACAGAAAGTAAAGCTTCTGGCATTCAATACAGTTTGTAGCAGTGGCTCTTAAATAAACAACAACA 9690
AACAAAGTCAAACTTGGGTGTATAGACCCACTGTACAGGCAGAACTCAAATGGCTTACCTCTTGGGTGAGCCTTGAACACCAGAGATGATCTTAACTAAATGC 9804
TGCACACACTAGGACCACCCAGAGTTACAAGTGTCTTGGACAGGGCCCTGCTCCCTTACGCTAAGTGAAGAGTGTGGTGGCTTGCCTTGCCTGAGGTGTGAGGCTAAAAC 9918
ACTGACCCACTGACCCCTGACTTGTAAAGACCTGTCTCACTTACCTTAATCTTGTAAAGCTTCAAATAAATACAAACATTTTGTAAAGGGTATCACCAGAAAT 10032
TAGGCCACGGCAGCATTATTTAAAGCTCAGGACCATGGATCTTCGAGGATTTTGGTAAAGTAAATAGGCTGGCGATCAGAAGGATAGGAGCTGCTCAGCTGCTGTAA 10146
CCTAACCTCCATCGGTGTGCGGACAGCCACAGTTCGACACCGTGGCTTCTCCTGTTGGGAACTATCCAAAGATTTCACTAACGAGACTCCTCATATGCAAAATTAAT 10260
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TGATATTAATAACAGTCACTTGTCTTACGCAAGGAGGCTTTTAAATTTTGGTTTAAAGTCAAGTTCACACTGAAATTAATTTT 10602
TAATTCACAGAGGGTGTGCTGCACTCAAGAGTTTGTGCAAGGACATTTTCAAAATTAATAATTTGTTTGAATTTAGAAAAAAA 10708

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Fig. 2. Nucleotide and translated amino acid sequences of the type 2 InsP₃ 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 p115 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 p16 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 p171, changing D at position 689 to H; the G at position 2397 was a T in p171 (silent change); the T at position 2604 was a C in p547-17 (silent change); the G at position 3283 was a T in p170, changing G at position 1013 to C; the G at position 3473 was a C in p170, changing G at position 1065 to A; the T at position 4013 was a C in p170, changing L at position 1256 to P; the C at position 4770 was a T in p165 (silent change); the G at position 4890 was an A in p165 (silent change); the G at position 4911 was an A in p165 (silent change); the G at position 5929 was an A in p115 (silent change); the G at position 7396 was a A in p115, changing V at position 2384 to I; and the A at position 8327 was a T in p16, changing E at position 2694 to V. In addition, clone p170 had an out-of-frame deletion from nucleotide 3311 to 3378. These sequence data are available from the EMBL/GenBank/DBJ databases under accession number X61677 ITPR2.

II	MSDKMSSFLYIGDIVSLYAEAGSNGFISTLGLVDDRCVVDPPEAGHLTNPKKFRDCLFKVCPMNRYSAQOKVYKAKOAKOGN	82
I	MSDKMSSFLHIGDICSLYAEGSTNGFISTLGLVDDRCVVDPPEAGHLTNPKKFRDCLFKVCPMNRYSAQOKVYKAKOAKOGN	82
II	HTEAALIKKIQHAAELIQONENSENRKLLGEIVKYSKVIQLLHKSRYLTVNKRPLALLEKNAMRVSLDAAGNEGSWFYIH	164
I	TTDAVLLNKLHHAADLEKKQNETENRKLGTVIQYGNVQLLHLKSNKYLTVNKRPLALLEKNAMRVSLDAAGNEGSWFYIQ	164
II	PFWLRESGDNVIVGDRVLLNPNVAGQPLHASSHQVLDNPGCNEVNSVNCNTSWKIVLFMKFSYREDVLKGGDVRVLFHAE	246
I	PFYKLRISIGDSVIVGDKVLLNPNVAGQPLHASSHQVLDNPGCNEVNSVNCNTSWKIVLFMKFSYREDVLKGGDVRVLFHAE	246
II	QEKFLTCDDYKFKQHIIFRTLRQSAFSAFSSAKLAEIETVNHDPCCGAGCGNSIFKFRHLAGNYLAAELNPFYRDAQNE	328
I	QEKFLTCDDYKFKQHVFLRITGRQSAFSAFSSAKLAEIETVNHDPCCGAGCGNSIFKFRHLAGNYLAAELNPFYRDAQNE	328
II	GKTVRDCGLPSTK KKHQAGEKINVTLSVPHGNDIASLLEFDATLQORADCLVPRNSYVLRHICTNTVTSTSTPIDTEE	409
I	FQPSVDPQDASRSRLNAQEKMYVSLVSVPEGNDISSIFELDPPTLRGGDSLVPNSYVLRHICTNTVTSTSTPIDTEE	410
II	ERPVMHKIGTCQTKEDKEAFAIVCVPLSEVRDLDFANDANKVLAITVKKLENGSITONERRRFTKLEDLIFVADVTRNGG	491
I	EKPVHLKIGTSLPKEDKEAFAIVCVPLSEVRDLDFANDANKVLAITVKKLENGSITONERRRFTKLEDLIFVADVTRNGG	492
II	DVLDVITKPKRERQKLMREQNLIAQVFGILKAPFKKAGESSMLREDELDQORYAPYKYVLRLLCYVLELHESQDDYRKNQEV	573
I	DVLEVVFSKPNREKQKLMREQNLIAQVFGILKAPFKKAGESSMLREDELDQORYAPYKYVLRLLCYVLELHESQDDYRKNQEV	573
II	IAKNFCVHQKQIGDILAEADITALLHNKRKLEKHITAAEITDTPVSLVRKNRERPFDLVLSDLCSNSTATLPVQELICKF	655
I	IAKQFQHQKQIGDILAEADITALLHNKRKLEKHITAAEITDTPVSLVRKNRERPFDLVLSDLCSNSTATLPVQELICKA	655
II	MLSPGNADILIQTKLVSMQVENPMESS ILPDDIDDEEVWLYWIDSNKPEHGKAIHRLAQEAREGTRADLEVLTYYRYQL	734
I	VLNPTNADILILETKLVSRFEGVSTGENALEAGEDEEVEWVFWDRSNKEIRSKVRELAQDAKGGQKEDRDLVSYRYQL	737
II	NLFARMCILDROYLAINOISTQLSVDLILRCVSDSESLPDLRASFCRLMLHHVDRDPQESVVPVRYARLWTEIPTKITHFY	816
I	NLFARMCILDRYLAINEISGLQDVLILRCVSDSESLPDLRASFCRLMLHHVDRDPQEQVTPVKYARLWSEITPKIAHIDDY	819
II	DSITDSSRNDHRRKFAITMEFVEEYLVKVVNPPFPGDKKKNLTFEVVHLARNLIYFGFYSFSELLRLTRTLAALDIVQA	898
I	DS SGASRDEIKERFAQTMEFVEEYLRDVVVCQRFPPSDEKKNLTFEVVHLARNLIYFGFYNFSDLRLTKLALALDQCVH	900
II	PHSSYFERLSKFD GSNVMKTIHGCVEMITQMVLSRGSIFPVSVPDAQPSVHPSKQASPGEOEDVTVMOTKIKVIELLO	978
I	TIFFISLTKGEEENKGS NVMSRHGVELTIVVL RGGGFLLPMTMAAPEGVKNQAEPE EKEDIHVMTKLIKIEILQ	979
II	FILSVRLDYRISYMLSIYKKEFGED NDCGNDPSASCTPETLPLSALVP DIDEIAAAQETMFAGRKKEKTPVQLDDEGGRT	1056
I	FILNVRDYRISCLLCIFKREFDESNSQSSETS SGNSSQEGPS NVPGALDFEHIIEEQAEIGFGSEENTPLDLDHGGRT	1059
II	FLRVLIHLIMHDYAPLLSGLQLLFKHFQRQAEVLAQAFQVQLVSNQDDVNYKQIKADLDQLRLTVKESLWVEK SGSYE	1137
I	FLRVLIHLIMHDYAPLLSGLQLLFKHFQRQAEVLAQAFQVQLVSNQDDVNYKQIKADLDQLRSIWESELWVEKSGSPE	1141
II	NGDMGEGQAKGEEANEESNLLSPVQDCAKTPQIDSNKGNVYRIVKEILIRLSKLCVON KKCRNQHQRLKKNMGAAHSV	1215
I	PMD GASGENEHKTEEGTSKPLKHESTS SYNRYVVKELIRLSKLCVQESASVRKSRKQKQQLLRNMGAAHV	1213
II	VLDLLOIPYER TDEKMNEMVMDLAHTFLQNFRCRNPNQVLLHKLNLFLTPGLEAETHRHIFMNNHYLHCNEISERVVQHF	1296
I	VLELLOIPYERAEDTKMQEIMRLAHEFLQNFRCAGNQOALLHKLNLFLNPGGLEAVTMQHIFMNNHQLCSEINERVVQHF	1295
II	VHCIEITHGRNVYELRFLOTIVKADGKYVKKCDQMVNTELINGGDEVLIYNDRASFPILNMMCSERARGDESGLP AYHIT	1377
I	VHCIEITHGRNVQYIKFLOTIVKAEGRKIKKCDQMVNTELINGGDEVLIYNDRASFTLQMHRSERDRMDENSPFLMYHIT	1377
II	LVLELAACTEGKNVYTEIKCNLSLPLDDIVRVVTHDDCIPEVKKIAYNFVNHCVVDTEVEMKEIYTSNHHWKLFENFLVMDA	1459
I	LVLELAACTEGKNVYTEIKCNLSLPLDDIVRVVTHDDCIPEVKKIAYNFVNHCVVDTEVEMKEIYTSNHMMKLFENFLVDC	1459
II	RVCNTITDRKHADTFLEKCVTESVMNIVSGFFNSPFSNSTSLQTHQPVFIQLLQSAFRINYCTWPNPQAKASVESCTRALA	1541
I	RACNNTSDRKHADSVLEKYVTEIVMSIVITTFSSPFSQOSTLQTRQPVVQLLQGVFRVYHGNWMLPQAKASVESCTRALS	1541
II	EVAKNRGAIAIPVDLSQVNTLFMKNSSTVQRAAMGWRLSARSQPRFKEALGPAWDRNIEKLODVVASLEQOFSPPMQA	1623
I	EVAKRAIAIPVDLSQVNNFLKSH NIVQKTAAMNRLSARNAAARDSVL AASRDYRNIEERLQDIVSALEDRRLPVLQA	1621
II	EPFSLVDVLYSPLEPFGSDARIRC GAFMSKLIINTKKLM EKEEKIKIKIQLTIREMLEKDKDSFMEE	1691
I	ELSVLVDVLRPELFPENTDARKKCESGGFICKLIKHTKQLLENEEKLCIKVLQTLREMMTKDRGYGKQISIDELENAE	1703
II	SSTLRKILLNRYFKGDHS VGVNGPLS GAYAKTAQVGGGFTQDAD	1736
I	LPQPPEAENSTEELSPPLRQLEDHNRGEALRQILLVNRYGNIRPSGRRESLTSFGNGPLSPGGPSK PGGGGGSGST	1784
II	KTG ISMSDIOCLDKEGASELVIDVIVNTKNDRIFSEGILLGIALLEGNTOTONSFYQOLHEQKSEKFFKVLVDRMKA	1817
I	SRGENSLAEVQCHLDKEGASNLVIDLIMNASSDRVHEHSILLAIALLEGNTTIQHSFFCRLEDKSEKFFKVPVDRMKVA	1866
II	QKEIRSTVTTNIDLGSKKREEDSDLMALGPRMRVDRSSLLKHEGKMGQLTTEASSATSKAYCVYRRMMDPIDTHCPQOEG	1899
I	QKEIKRAITVNTSDLGNKKDEVDVDR PSRKAKEPTTQIEVEVRDQLEASAATRKAFITFRREADPD DRYQG EGT	1945
II	SA EKSAEVEVMSPAITIMRPIILRFLQLLCCENHNRELQNFRLRQNNKTNVNLVCETLOFLDCICGSTGGGLGLLYINEK	1980
I	QATTDKAKDDLEMSAVITIMQPIILRFLQLLCCENHNRLQNFRLRQNNKTNVNLVCETLOFLDCICGSTGGGLGLLYINEK	2027
II	NVALNQITLESLEYCOGCPHENQTCIATHESNGIDIILAILLSINPLGKYRMDLVLQKNNASKLLLAIHESRHDSENAE	2062
I	NVALNQITLESLEYCOGCPHENQNCIATHESNGIDIILAILINDINPLGKRRMDLVLKNNASKLLLAIHESRHDSENAE	2109
II	RILFNMRKELEVDMKNAYNOGLECNHGDEEGDDG VSPKDVGHNIYILAHOLARNKELLOMLKPGSDPEEGDEALKYVA	2143
I	RILYNMRKELEVVIKAYMQG EVEFEDGEGEDGAASPRNVGHNIYILAHOLARNKELQTMKPG QVVDGEALEFYA	2189
II	NHTAQIEIVRDRMTEQIVFVPPNICEFLTRKSKYVFNTRERDEQGSKNVDFFOOTEDLYNEMKOKKIRNPNALFVFSRH	2225
I	KHTAQIEIVRDRMTEQIVFVPPNICEFLTRKSKRIYVTTREDEQGSKNVDFFLRSEDLFNEMNWKLLRAQPVLYVGRN	2271
II	ISLWGSISFNLAVFINLAVLAFYFDDGDEGTLSPLFSALLVAVAICTSMLEFFFSKPVGIRPFLVSI MLRSIYTIGLGPT	2307
I	MSFWSSISFNLAVMNLLVAFYFPGVGRG GTLEPHWSGLLWAMLSLAVIALPKPHGIRALIASTILRLIESVGLQPT	2352
	M1 M2 M3	
II	LILIGAANLCKNIVFLVSVFVGNRGTFTRGYRAVILDMAFVLYHVAIVLVCMLGLFVHEFFYSFLFLDLVYREETLLNVIKSVT	2389
I	LELLGAEVNVCKIIFLMSFVGNCGTETRGYRAMVLDVEFLYRLYLICAMGLFVHEFFYSLLFLDLVYREETLLNVIKSVT	2434
III	RETLEFNVIKSVT	13
	M4 M5 M6	
II	RNGRSITLTAVALILVYLFSIIGFLFKDDFTMEVDRKNTPTVGTNDGVPTMTLTSMLGTCPE NCSPTIPSSNAAG	2468
I	RNGRPIILTAALALILVYLFSIVGYLFFKDDFLEVDRLPNETAGPETGESLANDFLYSDVCVETGENCTSPAPKEELLPV	2516
III	RNGRSITLTAALALILVYLFSIVGFLFKDDFLEVDRLPNHSTASPLGMPHGAAAFVD TCSGDKMDCVSGLSVPEVLE	93
	M7 M8	
II	EGGEDGI ERTCDTLLMCIVTVLNOGLRNGGGVGDVLRPSPKDEFLFAARVVYDLFFFIVIIIVNLIFGVIIIDTFADLRS	2549
I	EETEDG I ERTCDTLLMCIVTVLNSHGLRSGGGVGDVLRKPKSKEEPLFAARVIVDLEFFVILIVNLIFGVIIIDTFADLRS	2597
III	EDRELDSTERACDTLLMCIVTVMNHGLRNGGGVGDVLRKPKSDESLFPARVVYDLL	147
	M9	
II	EKQKKEIKLTKTCFICGLERDKFDNRTVSFEHHSKSEHNMWHYLYFIVLVKVDPTIEYTGPESYVAQMITEKNLQWFFPRMA	2631
I	EKQKKEIKLTKTCFICGLERDKFDNRTVTFEHHSKSEHNMWHYLYFIVLVKVDPTIEYTGPESYVAEMIRERNLQWFFPRMA	2679
II	MSLVSNEGDSSEONEIRNLQEKLESTMSLVKQLSGOLAEIKQEMTEQRKNKORLGLGNSNTPHENHMHPPH*	2701
I	MSLVSSEGEQNELRNQLQEKLESTMSLVKQLSGOLSELKQEMTEQRKNKORLGLGNSNTPHENHMHPPHQA*	2749

Fig. 3. Alignment of the amino acid sequence of the rat type 2 InsP₃ 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 InsP₃ 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.

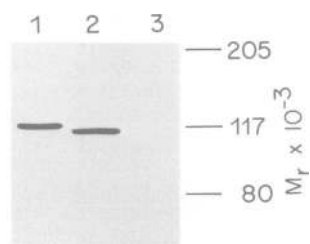


Fig. 4. Immunoblot analysis of proteins specified by the amino-terminal sequences of the type 1 and type 2 receptors expressed in COS cells. The amino-terminal 1081 and 1078 residues of the type 1 and type 2 InsP_3 receptors, respectively, were cloned into an expression vector fused to a sequence encoding 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 (pIP₃R-Stop1081, lane 1), type 2 InsP_3 receptor construct (pIP₃R2-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.

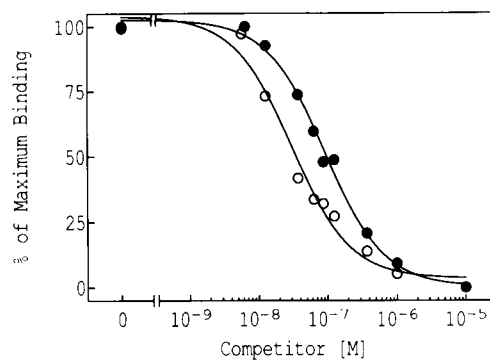


Fig. 5. Determination of the binding affinities of the ligand binding domains of the type 1 and type 2 InsP_3 receptors. Cytosol from COS cells transfected with constructs encoding the ligand binding domains of the type 1 (closed symbols) and type 2 InsP_3 receptors (open symbols) were used in binding-competition assays whereby the displacement of 3.3 nM [³²P] 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 $\text{Ins}(1,4,5)\text{P}_3$ receptors

Competitor (10 μM)	pIP ₃ R-Stop1081 (c.p.m./mg $\times 10^{-3}$)	Percentage of control	pIP ₃ R2-Stop1078 (c.p.m./mg $\times 10^{-3}$)	Percentage of control	Salmon sperm DNA
—	22.86 \pm 1.36	100.0	11.33 \pm 0.59	100.0	0.00 \pm 0.17
Inositol 1,4-bisphosphate	22.86 \pm 1.17	100.0	13.61 \pm 0.50	120.1	—
Inositol 4,5-bisphosphate	16.03 \pm 0.47	70.1	7.71 \pm 0.31	68.0	—
Inositol 1,4,5-trisphosphate	0.00 \pm 0.18	0.0	0.00 \pm 0.15	0.0	0.00 \pm 0.96
Inositol 2,4,5-trisphosphate	0.45 \pm 1.74	2.0	0.60 \pm 0.48	5.3	—
Inositol 1,3,4,5-tetrakisphosphate	8.68 \pm 1.20	38.0	5.05 \pm 0.93	44.6	—
Inositol 1,4,5,6-tetrakisphosphate	20.13 \pm 1.06	88.1	11.59 \pm 0.55	102.3	—
Inositol 1,3,4,5,6-pentakisphosphate	12.17 \pm 1.29	53.2	7.22 \pm 0.61	63.7	—
Inositol hexakisphosphate	17.86 \pm 0.49	78.1	9.46 \pm 1.11	83.5	—
Heparin (5 $\mu\text{g}/\text{ml}$)	2.62 \pm 0.94	11.5	3.58 \pm 1.28	31.6	—
Heparin (100 $\mu\text{g}/\text{ml}$)	0.00 \pm 0.50	0.0	0.00 \pm 0.78	0.0	—

³H- 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 pIP₃R-Stop1078 was 77% of that of pIP₃R-Stop1081 (2.95 and 3.81 $\times 10^6$ c.p.m. ¹²⁵I-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

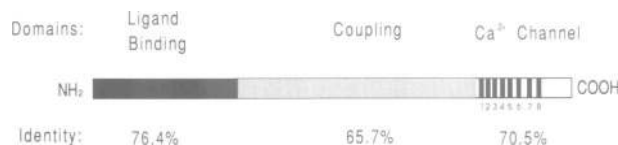


Fig. 6. Domain model of the InsP₃ receptors and sequence identities between the type 1 and type 2 InsP₃ receptors in the different domains. The three principal domains of the InsP₃ receptor are described on top (Mignery and Südhof, 1990). The eight putative transmembrane regions in the carboxy-terminal fourth of the receptors are numbered and their position indicated by vertical lines. The sequence identity between the two types of InsP₃ receptors in each domain is shown on the bottom.

differences are observed in the putative Ca²⁺ channel domain, particularly in the loops separating transmembrane regions. These differences suggest that the Ca²⁺ gating characteristics of the two receptors may also be different.

Our results demonstrate the presence of multiple types of InsP₃ 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 InsP₃. What is the biological relevance of the presence of different types of InsP₃ 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 InsP₃ receptors may be functionally similar but have different regulatory properties. This would result in differences of the properties of intracellular Ca²⁺ stores dependent on which InsP₃ receptors are expressed. This hypothesis is supported by the fact that the putative coupling domains of the InsP₃ 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 InsP₃ receptors could have different intracellular functions specified by different intracellular localizations. Currently it seems unlikely, although not excluded, that an InsP₃ 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 Ca²⁺ stores (Lechleiter *et al.*, 1991; Villa *et al.*, 1991).
3. Different types of InsP₃ receptors could have different intracellular functions analogous to the two forms of the ryanodine receptor. Ryanodine receptors, similar to InsP₃ receptors, release Ca²⁺ from intracellular stores. The ryanodine receptors from cardiac and skeletal muscle differ from each other in the coupling between membrane depolarization to Ca²⁺ 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 InsP₃ 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 InsP₃ receptor would support such a model.

All these hypotheses (independent of which will be proved to be correct) imply that Ca²⁺ signaling induced by InsP₃

is much more complex than that envisioned by a single type of receptor. Clearly Ca²⁺-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 InsP₃ receptors (oligonucleotide sequence: CAGCTGCAGGACGATGATGATGACCATGAAGAAGAA). Of the 15 clones isolated, most encoded the cerebellar InsP₃ receptor but two clones upon sequencing were found to be distinct from but homologous to the cerebellar InsP₃ 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 InsP₃ 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 InsP₃ 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 ³²P- and ³⁵S-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 InsP₃ receptor and ryanodine receptor.

Expression of the ligand binding sites of type 1 and type 2 InsP₃ receptors by transfection

pIP₃R2-Stop1078 is a mammalian expression vector in which the cytomegalovirus promoter drives the expression of the first 1078 amino acids of the type-2 InsP₃ 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. pIP₃2-Stop1078 was constructed by cloning the 2.45 kb *EcoRI*-*KpnI* 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 *KpnI*-*PstI* fragment from pI71 and by an oligonucleotide encoding the carboxy-terminal epitope. The corresponding type-1 InsP₃ receptor expression vector pIP₃R-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 InsP₃ (17 Ci/mmol) (NEN-Du Pont) except for the assays used for the determination of the binding affinities in which ³²P-labeled InsP₃ (155 Ci/mmol) 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-blot 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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References

- Berridge, M.J. (1990) *J. Biol. Chem.*, **265**, 9583–9586.
- Berridge, M.J. and Irvine, R.F. (1989) *Nature*, **341**, 197–205.
- Chadwick, C.C., Saito, A. and Fleischer, S. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2132–2136.
- De Camilli, P., Takei, K., Mignery, G.A. and Südhof, T.C. (1990) *Nature*, **344**, 495.
- Ely, J.A., Hunyady, L., Baukal, A.J. and Catt, K.J. (1990) *Biochem. J.*, **268**, 333–338.
- Ferris, C.D., Cameron, A.M., Bredt, D.S., Haganir, R.L. and Snyder, S.H. (1991a) *Biochem. Biophys. Res. Comm.*, **175**, 192–198.
- Ferris, C.D., Haganir, R.L., Bredt, D.S., Cameron, A.M. and Snyder, S.H. (1991b) *Proc. Natl. Acad. Sci. USA*, **88**, 2232–2235.
- Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N. and Mikoshiba, K. (1989) *Nature*, **342**, 32–38.
- Guillemette, G., Balla, T., Baukal, A.J. and Catt, K.J. (1988) *J. Biol. Chem.*, **263**, 4541–4548.
- Harootunian, A.T., Kao, J.P.Y., Paranjape, S., Adams, S.R., Potter, B.V.L. and Tsien, R.Y. (1991) *Cell Calcium*, **12**, 153–164.
- Irvine, R.F. (1990) *FEBS Lett.*, **263**, 5–9.
- Kozak, M. (1989) *J. Cell Biol.*, **108**, 229–241.
- Lechleiter, J., Girard, S., Clapham, D. and Peralta, E. (1991) *Nature*, **350**, 505–508.
- Mignery, G.A. and Südhof, T.C. (1990) *EMBO J.*, **9**, 3893–3898.
- Mignery, G.A., Südhof, T.C., Takei, K. and De Camilli, P. (1989) *Nature*, **342**, 192–195.
- Mignery, G.A., Newton, C.L., Archer, B.T., III and Südhof, T.C. (1990) *J. Biol. Chem.*, **265**, 12679–12685.
- Otsu, K., Willard, H.F., Khanna, V.K., Zorzato, F., Green and MacLennan, D.H. (1990) *J. Biol. Chem.*, **265**, 13472–13483.
- Palmer, S. and Wakelam, M.J.O. (1989) *Biochem. J.*, **260**, 593–596.
- Penner, R., Matthews, G. and Neher, E. (1988) *Nature*, **334**, 499–504.
- Perin, M.S., Fried, V.A., Slaughter, C.A. and Südhof, T.C. (1988) *EMBO J.*, **7**, 2697–2703.
- Perin, M.S., Fried, V.A., Stone, D.K., Xie, X.-S. and Südhof, T.C. (1991) *J. Biol. Chem.*, **266**, 3877–3881.
- Petersen, O.H., Gallacher, D.V., Wakui, M., Yule, D.I., Petersen, C.C.H. and Toescu, E.C. (1991) *Cell Calcium*, **12**, 135–144.
- Pietri, F., Hilly, M. and Mauger, J.-P. (1990) *J. Biol. Chem.*, **265**, 17478–17485.
- Ross, C.A., Meldolesi, J., Milner, T.A., Satoh, T., Supattapone, S. and Snyder, S.H. (1989) *Nature*, **339**, 468–470.
- Ross, C.A., Bredt, D. and Snyder, S.H. (1990) *Trends Neurosci.*, **13**, 216–222.
- Rossier, M.F., Capponi, A.M. and Vallotton, M.B. (1989) *J. Biol. Chem.*, **264**, 14078–14084.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Shears, S.B. (1991) *Cancer Cells*, **3**, 97–99.
- Streb, H., Irvine, R.F., Berridge, M.J. and Schulz, I. (1983) *Nature*, **306**, 67–69.
- Südhof, T.C., Lottspeich, F., Greengard, P., Mehl, E. and Jahn, R. (1987) *Science*, **238**, 1142–1144.
- Takei, K., Stukenbrok, H., Metcalf, A., Mignery, G.A., Südhof, T.C., Volpe, P. and De Camilli, P. (1992) *J. Neurosci.*, in press.
- Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T. and Numa, S. (1989) *Nature*, **339**, 439–445.
- Villa, A., Podini, P., Clegg, D.O., Pozzan, T. and Meldolesi, J. (1991) *J. Cell Biol.*, **113**, 779–791.
- Woods, N.M., Cuthbertson, K.S.R. and Cobbold, P.H. (1986) *Nature*, **319**, 600–602.
- Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N.M., Lai, F.A., Meissner, G. and MacLennan, D.H. (1990) *J. Biol. Chem.*, **265**, 2244–2256.

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