

Inositol 1,4,5-trisphosphate receptors: Distinct neuronal and nonneuronal forms derived by alternative splicing differ in phosphorylation

(inositol phospholipid/diacylglycerol/cAMP-dependent protein kinase/protein kinase C/calcium/calmodulin-dependent protein kinase)

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Contributed by Solomon H. Snyder, January 4, 1991

ABSTRACT We have identified two distinct transcripts of inositol 1,4,5-trisphosphate receptor by using the PCR on first-strand cDNAs from various rat tissues. The longer form, corresponding to the previously cloned adult rat brain inositol 1,4,5-trisphosphate receptor, contains a 120-nucleotide insert between the two cAMP-dependent protein kinase phosphorylation consensus sequences. The shorter form (lacking the insert) predominates in fetal brain and peripheral tissues and appears to represent a nonneuronal receptor, whereas the longer form is found in adult brain and appears to be exclusively neuronal. The phosphorylation kinetics by cAMP-dependent protein kinase and the phosphopeptide maps differ for inositol 1,4,5-trisphosphate receptors purified from tissues predominantly expressing different forms of the transcript.

The inositol phospholipid second messenger pathway involves the formation of inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol from the membrane phospholipid phosphatidylinositol bisphosphate (1). IP₃ acts intracellularly to release calcium from a subpopulation of the endoplasmic reticulum (1–3), whereas diacylglycerol remains membrane-associated and stimulates protein kinase C (4). The IP₃ receptor (IP₃R) protein has been purified to homogeneity (5). When the purified receptor is reconstituted into liposomes, it mediates an IP₃-dependent calcium flux, indicating that the receptor protein possesses a calcium channel as well as an IP₃ recognition site (6). The IP₃R is phosphorylated by cAMP-dependent protein kinase (PKA), diminishing the potency of IP₃ in releasing calcium from brain microsomes (7).

The primary sequences of mouse (8) and rat (9) brain IP₃Rs have been determined. The cloned cDNAs code for a 313-kDa protein with seven or eight-membrane-spanning domains near the C-terminal end (8–10), and two PKA consensus phosphorylation sites (Baa-Baa-Xaa-Ser, where Baa is a basic amino acid) in the midportion of the sequence. Mutational analysis of the expressed rat receptor suggests that the IP₃ binding site is at the N-terminal end and that binding of IP₃ causes a conformational change that presumably activates channel opening (10). In rat cDNA clones, Mignery *et al.* (9) detected a 45-nucleotide putative splice sequence in the N-terminal third of the protein.

We report herein identification of distinct neuronal and nonneuronal forms of the IP₃R. The neuronal form contains a 120-nucleotide insert, located between the two PKA phosphorylation consensus sequences, that is absent in nonneuronal tissue. We also report differences in PKA phosphory-

lation patterns for IP₃Rs derived from tissues enriched in mRNAs for the different forms of the receptor.

MATERIALS AND METHODS

Materials. [γ -³²P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq) was purchased from DuPont/NEN. Restriction enzymes, DNA polymerases, and modifying enzymes were purchased from Boehringer Mannheim and BRL. The Fast Track kit for poly(A)⁺ mRNA isolation and the cDNA Cycle kit for first-strand cDNA synthesis were purchased from Invitrogen (San Diego). Thermolysin was obtained from Boehringer Mannheim. All other chemicals were purchased from Sigma.

RNA Samples. Poly(A)⁺ RNA was isolated from rat tissue and cells by using oligo(dT)-cellulose affinity chromatography directly after homogenization and proteinase K treatment (Invitrogen Fast Track kit).

cDNA Synthesis and the PCR. First-strand cDNA was synthesized using avian myeloblastosis virus-reverse transcriptase and 1 μ g of poly(A)⁺ mRNA with random-hexamer DNA primers (Invitrogen cDNA Cycle kit). Oligonucleotide primers for the PCR were 5'-CCG-GAA-TTC-GGT-TCA-TCT-GCA-AGC-TAA-TAA-AAC-3' [bases 4852–4876 (9) plus the 5'-terminal *Eco*RI site] and 5'-CCG-GAA-TTC-AAT-GCT-TTC-ATG-GAA-TAC-TCG-GTC-3' [complementary to bases 5448–5472 (9) plus the 5'-terminal *Eco*RI site]. [Nucleotide numbering is based on rat cDNA sequence reported by Mignery *et al.* (9), modified such that the adenosine of the initiating ATG is position 1. One difference in sequence is noted in the text.] Each reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 0.1% glycogen, oligonucleotide primers at 50 μ g/ml, all four dNTPs (each at 5 mM), and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer/Cetus) in 50 μ l, with 15% of the first-strand cDNA products. After an initial cycle of 4 min at 94°C, the reaction was cycled 36 times with 60 sec at 62°C, 60 sec at 72°C, and 60 sec at 94°C. The reaction was completed with one cycle of 72°C for 5 min.

Subcloning. PCR-amplified DNA for sequencing was purified by electrophoresis on 1.2% agarose gels. Bands were visualized with ethidium bromide and excised from the gel. DNA was extracted using GeneClean (Bio 101, La Jolla, CA), cut with *Eco*RI, and cloned into *Eco*RI-digested calf intestinal alkaline phosphatase-treated pBluescript (Stratagene).

DNA Sequencing. Plasmid DNA was isolated by alkaline lysis. Double-stranded DNA was sequenced using Sequenase 2.0 (United States Biochemical) with primers complementary

to the T7 and T3 promoters in pBluescript and specific internal primers.

Southern Blot Analysis. PCR-amplified DNA was electrophoresed on 2% agarose gels, transferred to Immobilon membranes (Millipore), and probed with a ³²P-labeled oligonucleotide corresponding to bases 5077–5122 (9), which is within the alternative splice insertion.

Phosphorylation by PKA. The catalytic subunit of PKA was purified from bovine heart as described (11). IP₃R protein was purified from cerebellum and vas deferens as described (5, 6, 18). For kinetic studies, various amounts of purified IP₃R from vas deferens or cerebellum were incubated for 2 min with PKA at 30°C in a final volume of 100 μl. For phosphopeptide mapping, purified IP₃R was incubated for 60 min at 30°C. All reaction mixtures contained 10 mM MgCl₂ and 50 μM ATP (1 μCi/nmol), as well as PKA at 5 μg/ml. Protein concentrations were determined by Bradford protein assay (12) and by silver staining purified proteins electrophoresed on a 7% polyacrylamide gel containing SDS. Reactions were stopped by addition of 50 μl of 3× sample buffer for SDS/PAGE [6% (wt/vol) SDS/4.5 M 2-mercaptoethanol/15% (vol/vol) glycerol/150 mM Tris·HCl, pH 7.0/0.015% bromophenol blue]. After electrophoresis, the IP₃R protein was cut out of the gel and ³²P incorporation was determined by Cerenkov counting.

Phosphopeptide Maps. Phosphorylated IP₃R was excised from SDS/PAGE gels and digested with thermolysin (300 μg/ml) for 20 hr at 30°C. Phosphopeptide maps were determined as described (13).

RESULTS

Identification of the Splice Site in IP₃R cDNA. The primary sequences of the IP₃R obtained from mouse (8) and rat (9) brain cDNAs are highly conserved, with greater than 95% amino acid identity. In our efforts to clone the human IP₃R, we made use of adult human brain, fetal human brain, and human glioblastoma cDNA libraries. Overall, the human sequence was greater than 85% identical to the mouse and rat

sequences at the nucleotide level. However, we identified one cDNA clone in the glioblastoma library that differed from the adult human brain sequences by the absence of a 120-nucleotide stretch (bases 5077–5197).

We wondered whether the shorter sequence was specific to human cDNA or whether it reflected a phenomenon of RNA processing common to a number of species. Accordingly, we synthesized oligonucleotide primers (Fig. 1) flanking the area of deletion (the apparent splice site) and employed them in a PCR using first-strand cDNAs derived from fetal and adult rat brain RNA as templates. The PCR products migrated on an agarose gel as fragments of different lengths; the predominant product of the adult cDNA (long form) was 120 nucleotides longer than that of the fetal cDNA (short form). The amplified DNA fragments were subcloned into pBluescript and sequenced (Fig. 1). The sequence of the adult rat brain IP₃R fragment was homologous to that of the adult human brain, whereas the fetal rat brain IP₃R, 120 nucleotides shorter than the adult form, was similar to the sequence obtained from a glioblastoma cDNA library. The divergence occurred at nucleotide 5077, preserved the reading frame, and was preceded by the splice donor consensus sequence AG, suggesting that the two forms occur by alternative splicing of a single transcript. The corresponding 40-amino acid interval in the adult rat brain form of the receptor occurred in the putative cytoplasmic domain of the IP₃R and was located between two PKA phosphorylation consensus sequences (Ser-1589 and Ser-1756). Our adult rat brain sequence differed from the published sequence (9) by a glutamine inserted after Glu-1715; thus our subsequent amino acids differ by one position from the published sequence.

Identification of Short and Long Forms of IP₃R in Various Tissues. To explore the developmental expression of the two forms of the IP₃R message, we performed a PCR on first-strand cDNAs derived from mRNA extracted from brain at various developmental stages, by using primers that flanked the apparent alternative splice site (Fig. 2A). At early developmental stages, it was not convenient to isolate RNA from separate brain regions because of the limited quantity of

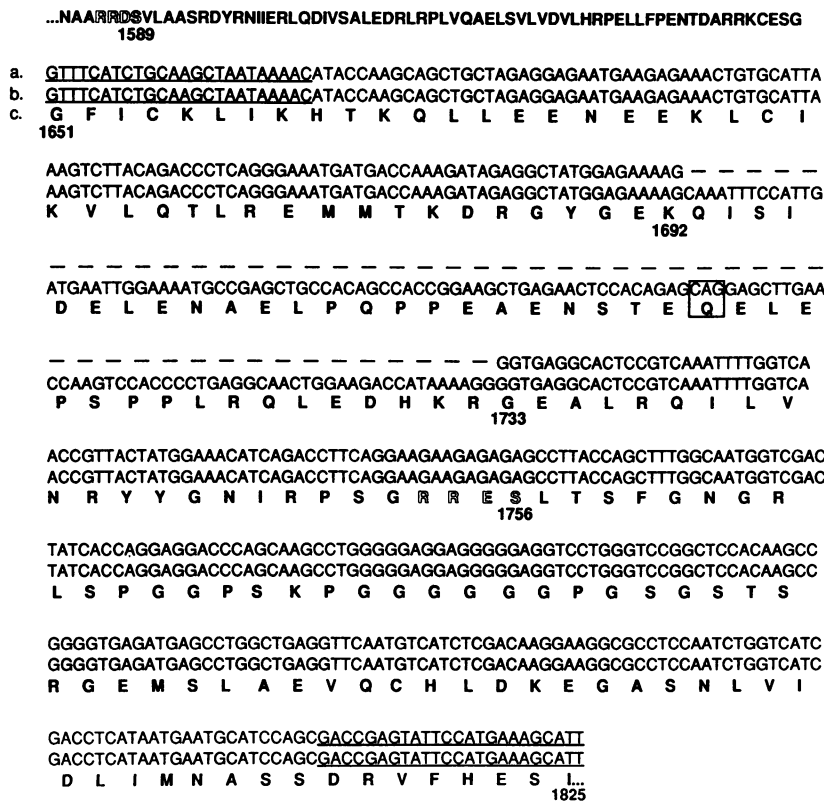


FIG. 1. Location of PKA phosphorylation consensus sequences and alternative splice site in the rat IP₃R primary sequence as determined by sequencing PCR-amplified first-strand cDNA from tissues expressing the short or the long forms of the IP₃R. Lines: a, nucleotide sequence of the short form derived by a PCR of first-strand cDNAs from vas deferens; b, nucleotide sequence of the long form derived by a PCR of first-strand cDNAs from cerebellum; c, amino acids, numbered corresponding to published sequence (9). Oligonucleotide primers for the PCR are underlined. PKA phosphorylation consensus sequence (Baa-Baa-Xaa-Ser) is shown by open letters. Amino acids 1692 and 1733 are brought together in tissues expressing the short transcript. Gln-1716, which is boxed, is absent in the reported rat cDNA sequence (9).

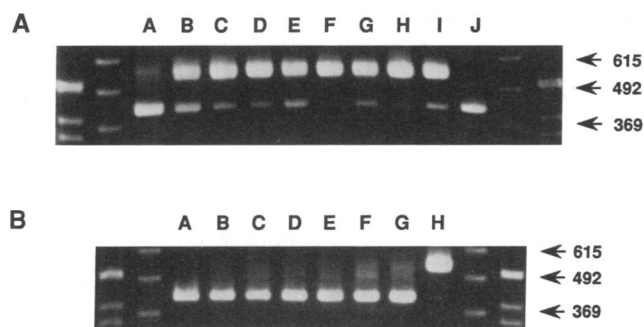


FIG. 2. PCR products from developmental and regional first-strand cDNAs. RNA was isolated from rat tissues at various developmental stages. First-strand cDNA was synthesized and used as template with primers flanking the insert site. The outermost two lanes on each end of the gel contain a 1-kilobase ladder and 123-base-pair markers. Molecular sizes are shown in base pairs. (A) Lanes: A, embryonic day 17 head; B, embryonic day 21 whole brain; C, 3-day-old cerebellum and cortex; D, 10-day-old cerebellum; E, 10-day-old cortex; F, 17-day-old cerebellum; G, 17-day-old cortex; H, adult cerebellum; I, adult cortex; J, C6 glioma cells. (B) Lanes: A, vas deferens; B, testis; C, thymus; D, lung; E, liver; F, spleen; G, adrenal; H, cerebellum (all from adult rat).

tissue; therefore, whole head, whole brain, or mixtures of cerebellum and cerebral cortex were used. At embryonic day 17, mRNA derived from whole rat head contained predominantly the short message. By embryonic day 21, both messages were evident in mRNA from whole rat brain. At postnatal day 3, mRNA extracted from a mixture of cerebellum and cerebral cortex (brain regions known to express high levels of IP₃R) showed a predominance of the long message. At day 10, mRNA was isolated separately from cerebellum and cerebral cortex. At days 10 and 17 and in the adult rat, cerebellum expressed almost exclusively the long transcript, whereas the cerebral cortex continued to express small amounts of the short transcript but predominantly the long transcript.

Since neurons develop primarily in the later fetal period of the rat, we suspected that the long form might represent a neuronal form, whereas the short form, which predominates in the fetus, might be a nonneuronal form. To evaluate this possibility, we conducted a PCR on first-strand cDNAs from RNAs isolated from various peripheral tissues (Fig. 2B). In vas deferens (a smooth muscle tissue with high levels of IP₃R), testis, thymus, lung, liver, spleen, and adrenal, we detected only the short form of the IP₃R. In addition, in a preparation from C6 glioma, a culture derived from cells of glial origin, we also observe only the short form of the receptor (Fig. 2A), supporting the hypothesis that the short form represents a nonneuronal type of IP₃R.

To confirm the identity of the PCR products, we synthesized an oligonucleotide corresponding to a sequence (bases 5077–5122) within the portion of the long message that was absent in the short transcript. We used this to probe Southern blots of PCR products from various developmental stages (Fig. 3A) and for various peripheral tissues (Fig. 3B). In all cases, the probe labeled only the band corresponding to the long form of the IP₃R.

Differential Phosphorylation of Neuronal and Nonneuronal IP₃Rs. Because the alternative splice sequence coded for amino acids forming a major portion of the sequence between the two putative PKA phosphorylation sites, we examined the phosphorylation patterns of the two forms of the receptor. Since the adult cerebellum contained exclusively the long form and the vas deferens contained exclusively the short form, we employed these two tissues for initial studies. By utilizing IP₃R protein purified to homogeneity from the two tissues, we evaluated phosphorylation by PKA with various

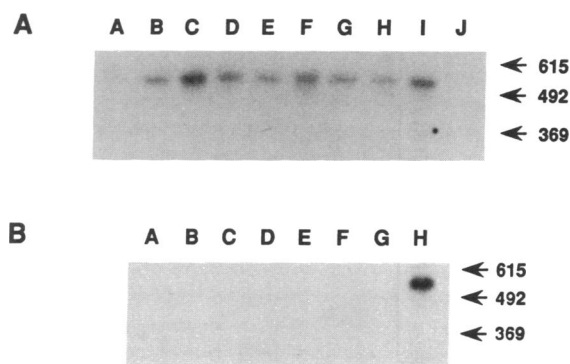


FIG. 3. Southern blot of PCR products. The DNA from gels in Fig. 2 was transferred to Immobilon. The blots were probed with a ³²P-labeled oligonucleotide corresponding to a portion of the insert. (A) Lanes: A, embryonic day 17 head; B, embryonic day 21 whole brain; C, 3-day-old cerebellum and cortex; D, 10-day-old cerebellum; E, 10-day-old cortex; F, 17-day-old cerebellum; G, 17-day-old cortex; H, adult cerebellum; I, adult cortex; J, C6 glioma cells. (B) Lanes: A, vas deferens; B, testis; C, thymus; D, lung; E, liver; F, spleen; G, adrenal; H, cerebellum.

concentrations of receptor protein (Fig. 4). Both receptors were stoichiometrically phosphorylated by PKA. However, IP₃R from vas deferens was phosphorylated more readily at lower substrate concentrations, displaying a K_m of 3.1 nM for the substrate which was about 20% that of the cerebellar receptor ($K_m = 17.5$ nM).

To determine the sites of phosphorylation of the two receptor preparations, we conducted two-dimensional phosphopeptide mapping (Fig. 5). The adult cerebellar receptor displayed two phosphopeptides (Fig. 5A). We have shown by peptide sequencing that the more basic of the two peptides, which contains about 90% of the radioactivity in this experiment, involved phosphorylation on Ser-1756, whereas the minor phosphopeptide corresponded to phosphorylation on Ser-1589 (14). By contrast, in the vas deferens, phosphorylation was almost exclusively on the less basic peptide, representing Ser-1589 (Fig. 5B).

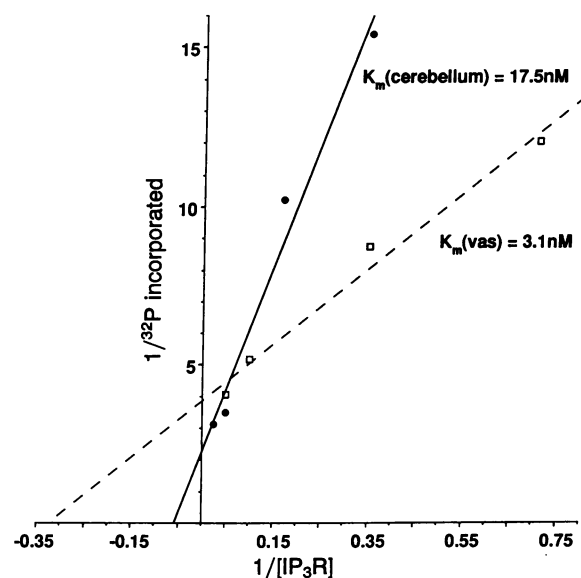


FIG. 4. Phosphorylation kinetics of IP₃R purified from rat cerebellum and vas deferens. Various quantities of IP₃R, purified as described (5, 18), were incubated with PKA (5 μg/ml) for 2 min at 30°C. This experiment was repeated three times with similar results. ^{1/32}P incorporated is shown as 1/(cpm × 10⁻³) and 1/[IP₃R] is shown as nM⁻¹.

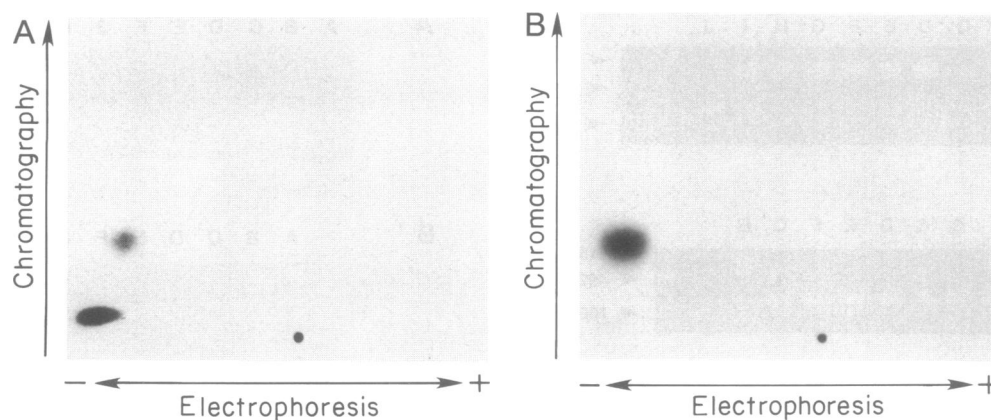


FIG. 5. Two-dimensional phosphopeptide maps of purified IP₃R from rat cerebellum and vas deferens phosphorylated by PKA. After phosphorylation of the purified IP₃R protein, proteins were electrophoresed by SDS/PAGE and stained. The bands corresponding to the IP₃R were excised and digested with thermolysin. The resultant peptides were lyophilized and subjected to electrophoresis and chromatography, as described (12). This experiment was repeated three times with similar results. (A) Cerebellar IP₃R. (B) Vas deferens IP₃R.

DISCUSSION

In this study, we have shown that there are distinct short and long transcripts of the IP₃R, most likely derived by alternative splicing. The short form is characteristic of nonneuronal tissues, whereas the long form appears to be predominantly, if not exclusively, neuronal. Purified IP₃R from tissues expressing the short or the long form differ in their kinetics and patterns of phosphorylation by PKA.

We have found the long form of the IP₃R transcript only in brain tissue but not in C6 glioma cells, suggesting that it is a neuronal form; however, it is not yet known which form is present in peripheral neurons and spinal cord. Within the brain, the adult cerebellum expresses almost exclusively the long form, and the adult cerebral cortex has a small amount of the short form as well. This can be explained by the extremely high level of expression of the IP₃R in Purkinje cells of the cerebellum, so that the short form, presumably derived from glia, would be present in a relatively smaller amount. By contrast, in the cerebral cortex where neuronal IP₃R expression is lower, transcripts from glia would contribute relatively more of the total message. All peripheral tissues examined, including several with high levels of IP₃R expression, contain exclusively the short transcript.

Several proteins have been shown to differ in neuronal and nonneuronal forms by an alternative splicing event. One example involves the *src* oncogene in which the neuronal form contains two distinct splice sites in tandem, designated N1 and N2 (15, 16). When both alternative splices are expressed, 17 amino acids are encoded that include a serine producing a potential phosphorylation site (16). Prior to the identification of N2, it had been shown at the protein level that neuronal *src* displays a serine phosphorylation site that is absent in nonneuronal *src* (17).

In the current study, we have found that purified IP₃R from adult cerebellum (long form) and vas deferens (short form) have different K_m values for substrate for phosphorylation by PKA. In addition, the two forms of the receptor are predominantly phosphorylated at different sites. We have purified the vas deferens receptor to homogeneity (18). Its molecular weight, affinity for IP₃, and most other properties are essentially the same as the cerebellar receptor. We cannot exclude the possibility that other factors, such as other alternative splicing events, are responsible for these different phosphorylation patterns. However, one explanation of these differences in phosphorylation is that they relate to the insert described herein, perhaps because it alters relative accessibility of the serines.

Mutational analysis of the IP₃R by Mignery and Südhof (10) suggests three main functional domains: an N-terminal IP₃ binding domain, a C-terminal calcium channel domain, and a long intermediate region containing the PKA sites and the alternatively spliced region described herein. Mignery and Südhof (10) term this intermediate region the "coupling domain" and suggest that it conveys a conformational change induced by IP₃ binding that leads to channel opening. Thus, the PKA sites might be involved in regulation of this coupling. Indeed, PKA phosphorylation of the cerebellar IP₃R decreases the effectiveness of IP₃ in mediating ⁴⁵Ca²⁺ flux in microsomes (7).

What might be the functional consequences of the alternative splicing? If splicing alters phosphorylation, then coupling efficiency of the receptor may be affected. Phosphorylation of the cerebellar receptor by PKA diminishes the effectiveness of IP₃ in mediating ⁴⁵Ca²⁺ flux in microsomes (7). We do not yet know the manner in which the IP₃-induced calcium flux in the vas deferens IP₃R is affected by phosphorylation, but we speculate that it may differ from that of the cerebellar receptor.

Our preliminary phosphopeptide maps of IP₃R derived from testis and C6 glioma suggest that other splicing events might also contribute to the overall phosphorylation pattern. Independently, several distinct splice sites in the vicinity of the 120-nucleotide site as well as the splice site described herein have been observed in mouse IP₃R (T. Nakagawa, H. Okano, and K. Mikoshiba, personal communication).

We have shown (19) that cerebellar IP₃R is also phosphorylated by protein kinase C and calcium/calmodulin-dependent protein kinase II. We have not yet ascertained whether these phosphorylations differ in the two IP₃R forms.

The alternative splicing might also affect properties of the receptor other than phosphorylation. The binding of IP₃ to its receptor is inhibited by nanomolar concentrations of calcium in membrane preparations and in crude detergent-solubilized cerebellar receptor, whereas IP₃ binding to purified IP₃R is not influenced even by high concentrations of calcium (5, 20). Inhibition by calcium of IP₃ binding to purified receptor can be restored by a membrane protein extract designated calmedin (5, 21). In membrane preparations and crude detergent-solubilized preparations of the vas deferens, calcium is only a weak inhibitor of IP₃ binding (18). The inability of calcium to inhibit IP₃ binding in the vas deferens appears to be due primarily to a lack of calmedin activity in the vas deferens (18). However, it is conceivable that there is some intrinsic difference in the ability of the vas deferens and cerebellar receptors to interact directly with calcium.

Yet another property of the IP₃R that might be altered by the splicing is the interaction with ATP. ATP alters the ability of IP₃ to mediate calcium flux from purified reconstituted receptors at a site unrelated to phosphorylation (22). When ATP concentrations are reduced below physiologic millimolar levels, IP₃ becomes more potent in influencing calcium flux. A putative ATP recognition site occurs close to the area of alternative splicing at amino acids 1774–1783.

We thank R. Haganir, R. Reed, and L. Brody for helpful discussion and Sylvia Gärtner and Uta Eichelsbacher for expert technical assistance. We thank Dawn Dodson and Jackie Shaw for assistance in preparing the manuscript. This work was supported by U.S. Public Health Service Grants MH-18501 and DA-00266 and Research Scientist Award DA-00074 to S.H.S., Grant MH-43040 and a National Alliance for Research in Schizophrenia and Depression Young Investigator Award to C.A.R., Predoctoral Fellowship MH-10018 to C.D.F., Training Grant GM-07309 to S.K.D., a grant from the International Life Sciences Institute, and a gift from Bristol-Myers Squibb. C.A.R. is a Pew Scholar in the Biomedical Sciences.

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