Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 4265–4269, May 1992
Neurobiology

Three additional inositol 1,4,5-trisphosphate receptors: Molecular cloning and differential localization in brain and peripheral tissues

(second messengers/intracellular calcium/cerebellar Purkinje cells/T lymphocytes/plasma membrane)

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Contributed by Solomon H. Snyder, January 6, 1992

ABSTRACT **Three inositol 1,4,5-trisphosphate receptor (IP_3R) cDNAs, designated IP_3R-II, -III, and -IV, were cloned from a mouse placenta cDNA library. All three display strong homology in membrane-spanning domains M7 and M8 to the originally cloned cerebellar IP_3R-I, with divergences predominantly in cytoplasmic domains. Levels of mRNA for the three additional IP_3Rs in general are substantially lower than for IP_3R-I, though in the gastrointestinal tract the levels of IP_3R-III may be comparable to IP_3R-I. Cerebellar Purkinje cells express at least two and possibly three distinct IP_3Rs, suggesting heterogeneity of IP_3 action within a single cell.**

Many cellular responses to neurotransmitters, hormones, and growth factors involve release of intracellular Ca^{2+} by inositol 1,4,5-trisphosphate (IP_3) (1–3) acting at specific receptors located on subcompartments of intracellular membranes, including endoplasmic reticulum (4–6) and, in some cells, plasma membrane (7). The purified IP_3 receptor (IP_3R) (8) has been shown by reconstitution experiments to incorporate the associated Ca^{2+} channel (9). IP_3R proteins purified to homogeneity from cerebellum (8), vas deferens (10), and aortic smooth muscle (11) all appear biochemically similar if not identical. Molecular cloning studies, however, have revealed heterogeneity. Full-length cDNAs for the cerebellar IP_3R have been cloned from mouse (12, 13) and rat (14, 15), and partial sequence has been obtained from human (16). One alternatively spliced form of the receptor involves insertion of 15 amino acids in the N-terminal portion (14, 17). Another involves 40 amino acids inserted into the coupling domain of the receptor between two consensus cyclic AMP-dependent phosphorylation sites (17, 18). This latter insert appears to be specific to neurons (18). Differential localizations or phosphorylations of some of these alternatively spliced forms of IP_3R suggest different functions (17, 18).

Receptor heterogeneity can also arise from different genes. Recently, evidence for IP_3Rs derived from different genes has been reported in rat (19) and mouse (16). Südhof *et al.* (19) obtained the complete coding sequence for one of these receptors and showed that expression of the N-terminal portion is associated with high-affinity IP_3 binding. We now report independent molecular cloning of three IP_3R-related sequences from mouse.‖ Polymerase chain reaction (PCR) analysis and *in situ* hybridization show differential tissue localizations. At least two IP_3R sequences are expressed in Purkinje cells, indicating heterogeneity of IP_3 actions within a single cell.

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METHODS

Library Screening. To identify IP_3R-related cDNAs, a cDNA library derived from mouse placenta (18th day of gestation) was screened at low stringency with a cDNA probe constituting 6.5 kilobases (kb) of the 3′ end of the rat IP_3R sequence. The probe was labeled by the random primer reaction (20). This 6.5-kb clone was obtained by screening a rat brain cDNA library (Stratagene, a36501) with a 1.8-kb PCR product generated using primers (5′-1, bases 5908–5931; 3′-1, complementary to bases 7713–7737) derived from the original mouse IP_3R sequence (12). A placenta library was chosen for low-stringency screening, because screening brain libraries with the same probe yielded such a large number of clones corresponding to the original IP_3R that it was difficult to detect new ones. Two replica filters were incubated with rat IP_3R cDNA probe under low-stringency conditions [6× standard saline/phosphate/EDTA (SSPE) with 10% formamide at 42°C], and a third replica filter was incubated with the same probe under high-stringency conditions (6× SSPE with 50% formamide at 42°C). Low-stringency washes contained 1× SSPE and 10% formamide at 42°C, and high-stringency washes contained 1× SSPE and 50% formamide at 42°C. Only signals that were doubly positive at low stringency and negative at high stringency were chosen for further characterization. After plaque purification, Southern blots of phage DNA digested with *Eco*RI were probed in the same fashion at high and low stringency. Four clones were positive at low stringency and negative at high stringency.

Inserts from these clones were subcloned into the pBluescript vector (Stratagene) and sequenced on both strands by using plasmid primers or specific internal primers. The four clones were designated M-1, M-3, M-5, and M-7. Further sequence analysis (performed by Betsy Nanthakumar at the Johns Hopkins Genetics Core Facility using an Applied Biosystems automated sequencing apparatus) showed that the M-3 sequence was entirely contained within the sequence of clone M-7. Data from clones M-1, M-5, and M-7 are presented here. The 2400-base sequence (clone M-7) appears to be a mouse homologue of rat IP_3R-III of Südhof *et al.* (19), and the 600-base sequence (clone M-5) appears to be a mouse homologue of human IP_3R-III of Südhof *et al.* (19).

Northern Blot Analysis. Total RNA was extracted from mouse placenta (19th day of gestation) by the cesium chloride centrifugation method (21). High molecular weight-enriched total RNA (20 μg per lane) was electrophoresed in a dena-

Abbreviations: IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor.

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‖The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M90087–M90089).

turing formaldehyde/agarose gel and transferred to nitrocellulose (21). Probes were inserts from the plasmid subclones described above (IP_3R-II, -III, and -IV), labeled to high specific activity by the random primer reaction. Hybridization and washing employed high-stringency conditions as described above. With these conditions none of the probes crossreacted appreciably with plasmid DNA corresponding to the original IP_3R (IP_3R-I). After extensive washing, blots were exposed to film for 7 days.

PCR Blot Experiments. Since Northern blot experiments indicated that the IP_3Rs were expressed at relatively low levels, we used PCR amplification of first-strand cDNA reverse-transcribed from RNA to screen for tissue expression of the IP_3Rs. Total RNA was extracted from various tissues by the acid guanidinium/phenol/chloroform extraction method (22) and quantified by absorbance spectrometry. Equal amounts of RNA from the different tissues were converted to first-strand cDNA by RNase H^- reverse transcriptase (BRL) with oligo(dT) primer. PCR was performed using ≈5% of this reaction product as template with primers specific for each of the three clones. Conditions were adjusted so that a single band was amplified in each reaction and there was no cross amplification of different messages.

Primers for IP_3R-II were M7-5′-2 (5′-GGC-ACA-CCC-TCT-CCA-TGT-GGC-AGG-3′) and M7-3′-2 (5′-TTC-CTG-TCT-CGC-TGG-ACC-CCA-GTG-3′). These primers were used to amplify a 200-base-pair (bp) segment within the 3′ untranslated portion of IP_3R-II. Primers for IP_3R-III were M5-5′-3 (5′-CAC-GGA-GCT-GCC-ACA-TTT-ATG-GGC-3′) and M5-3′-3 (5′-TCC-TCA-GTC-CGT-GGT-TCA-TGA-CGG-3′). These primers were used to amplify a 160-bp segment from the nonconserved region of IP_3R-III. Primers for the putative IP_3R-IV were M1-5′-1 (5′-GCC-CCG-TGG-CTC-TTC-TCC-CTG-3′) and M1-3′-1 (5′-GCA-GTA-CTC-ATC-TGG-GGG-CAT-GAT-3′). These primers were used to amplify a 120-bp segment within the nonconserved region of IP_3R-IV. Primers for actin were 5′-1 (5′-GTT-TGA-GAC-CTT-CAA-CAC-CCC-3′) and 3′-1 (5′-GTG-GCC-ATC-TCC-TGC-TCG-AAG-TC-3′). These amplified an ≈350-bp segment of mouse actin cDNA.

In each reaction, primers for actin were included so that coamplification of actin could be used as a control to ensure equal amounts of template and equal efficiencies of amplifications in all the samples. In addition, all samples were amplified using the same master mixture, which contained all components of the reaction except for the template. The amounts of templates were adjusted to yield equal amplification of actin. Control reactions in the absence of template yielded no detectable product. After an initial denaturation step of 94°C for 5 min, the cycle was 68°C annealing for 2 min, 72°C extension for 2 min, 94°C denaturing for 1 min, for a total of 20–32 cycles, with a final extension step for 10 min. The number of cycles was adjusted to give a barely visible product in at least one lane for each of the clones. This required 20–23 cycles for IP_3R-III and -IV, and 30–32 cycles for IP_3R-II.

A portion of the reaction mixture was run in an agarose gel and transferred to nitrocellulose. Blots were incubated with probes under hybridization conditions specific for each of the clones. While PCR is inherently a nonquantitative technique, standardization by coamplifying actin provides a semiquantitative screen of message expression in different tissues.

***In Situ* Hybridization.** To localize the cellular sites of expression, we used *in situ* hybridization with oligonucleotide or RNA probes. *In situ* hybridization with oligonucleotide probes was performed as described (23). Since IP_3R-II and IP_3R-IV were so similar in the membrane-spanning regions, we used probes specific for the nonconserved region for IP_3R-IV localization. Probes were M1-4 (5′-TGA-CAG-AGG-GGA-CCC-CGG-CAC-ACC-ACA-GCA-GGG-AGA-AGA-GCC-ACG-3′) and M1-5 (5′-GGC-ATG-ATC-CAG-AGT-GGA-GAA-GGC-ACC-CGA-GAA-AGG-GTA-TGG-GAG-3′).

For *in situ* hybridization with RNA probes, we used the method of Simmons *et al.* (24) with minor modifications (probes were not predigested and sections were not pretreated with proteinase K). Probes were transcribed in both the sense and the antisense orientation.

RESULTS

Sequence Comparisons and Message Size of IP_3Rs. Sequences of the three IP_3Rs identified in this work and IP_3R-I, the originally cloned mouse P400 cerebellar form (12), all display strong homology in membrane-spanning domains M7 and M8 and near the C terminus (Fig. 1). There is considerable divergence in the cytoplasmic loop between M6 and M7. The sequence of IP_3R-IV is virtually identical to that of IP_3R-II in M7 and M8, with absolute identity for a stretch of nearly 100 amino acids and near-absolute identity in this region at the nucleotide level. However, IP_3R-IV and IP_3R-II diverge substantially in the cytoplasmic loop between M6 and M7. Percentages of amino acids identical for IP_3R-II, -III, and -IV compared with IP_3R-I were 70%, 71%, and 75.8%, respectively.

To estimate message size, we conducted Northern blot analysis of RNA from mouse placenta, the tissue from which the clones were obtained (Fig. 2). The mRNAs for IP_3R-II, -III, and -IV were each about 10.5 kilobases, essentially the same as for IP_3R-I. On shorter exposure we observed two bands for IP_3R-I and IP_3R-II, which might reflect differential polyadenylylation sites, similar to the results of Südhof *et al.* (14, 19).

Tissue Distribution of IP_3Rs by PCR Analysis. PCR analysis revealed widespread expression for IP_3R-II, -III, and -IV but differential tissue localizations for the various receptor forms (Fig. 3).

Message for IP_3R-II was present at relatively low levels, since 30 cycles were necessary to produce visible products. Highest expression was in brain (Fig. 3), lung, and placenta, with product also visible in samples derived from liver, kidney, testes, and spinal cord. Expression in the cerebellum was greater than that in the forebrain.

IP_3R-III displayed an apparently wider distribution (Fig. 3). Highest levels appeared to be in the brain and gastrointestinal tract, with message also present in lung, liver, kidney, testes, thymus, spleen, placenta, and cerebellum.

Message for the presumptive IP_3R-IV also appeared to have wide distribution, with highest levels in the brain, spinal cord, and testes; slightly lower levels in lung, kidney, gastrointestinal tract, and placenta; and much lower levels in liver, thymus, and spleen. Expression appeared similar in cerebellum and forebrain.

Localization by *in Situ* Hybridization. Consistent with the low levels of IP_3R-II detected in PCR experiments in brain and peripheral tissues, *in situ* hybridization with the IP_3R-II probe in 21-day mouse embryo and adult sagittal brain sections revealed no specific label (data not shown).

IP_3R-III message was detected in whole fetal mouse sections, with high densities in the gastrointestinal tract (Fig. 4*A*). The label appeared localized predominantly to the smooth muscle layer, with very low signals in epithelial layers. Sense-orientation probes labeled to the same specific activity yielded no specific hybridization (Fig. 4*B*). In the brain, IP_3R-III signals were faint, with the suggestion of silver grains localized to Purkinje cells of the cerebellum (data not shown).

For the presumptive IP_3R-IV we utilized two oligonucleotide probes directed to the sequence in which IP_3R-IV diverges from the other IP_3Rs. In whole fetal sections, we have not detected specific hybridization (data not shown). In

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2201 DRTMEQIVFPVPSICEFLTKESKLRIYYTTERDEQGSKINDFFLRSEDLF 2250 IP3R-I
   1                     RESKYRVFNTTERDEQGSKVNDFFQQTEDLY 31   IP3R-II

2251 NEMNWQKKLRAQPVLYWCARNMSFWSSISFNLAVLMNLLVAFFYPF KGV 2299 -I
  32 NEMKWQKKIRNNPALFWFSRHISLWGSISFNLAVFINLAVALFYPFGDDG 81   -II
                              M1

2300 RGGTLEPHWSGLLWTAMLISLAIVIALPKPHGIRALIASTILRLIFSVGL 2349 -I
  82 DEGTLSPLFSALLWVAVAICTSMLFFFSKPVGIRPFLVSIMLRSIYTIGL 131  -II
               M2                              M3

2350 QPTLFLLGAFNVCNKIIFLMSFVGNCGTFTRGYRAMVLDVEFLYHLLYLL 2399 -I
 132 GPTLILLGAANLCNKIVFLVSFVGNRGTFTRGYRAVILDMAFLYHVAYVL 181  -II
       M3                    M4                    M5

2400 ICAMGLFVHEFFYSLLLFDLVYREETLLNVIKSVTRNGRSIILTAVLALI 2449 -I
 182 VWMLGLFVHEFFYSFLLFDLVYREETLLNVIKSVTRNGRSIILTAVLALI 231  -II
       M5                                    M6

2450 LVYLFSIVGYLFFKDDFILEVDRLPNETAVPETGESLANDFLYSDVCRVE 2499 -I
 232 LVYLFSIIGFLFLKDDFTMEVDRLKNRTPVTG NDGVPTMTLTSMLGTCP 280  -II
   1       M6             RARRPHGAAT             FMGTCSGD 18   -III
   1                                APWLFSLLWCAGVPSVT 17   -IV

2500 TGENCTSPAPKEELLPAEETEQDKEHTCETLLMCIVTVLSHGLRSGGGVG 2549 -I
 281 K ENCSPTIPSSN AAGEGGEDGIERTCDTLLMCIVTVLNQGLRNGGGVG 328  -II
  19 KME CVSEVSVPEILEEDEELDSTERACDTLLMCIVTVMNHGLRNGGGVG 67   -III
  18 SDNSHTLSRVPSPLWIMPPD        EYCLMCIVTVLNQGLRNGGGVG 59   -IV
                                        M7

2550 DVLRKPSKEEPLFAARVIYDLLFFFMVIIIVLNLIFGVIIDTFADLRSEK 2599 -I
 329 DVLRRPSKDEPLFAARVVYDLLFFFIVIIIVLNLIFGVIIDTFADLRSEK 378  -II
  68 DILRKPSKDESLFPARVVYDLLFFFIVIIIVLNLIFGVIIDTFADLRSEK 117  -III
  60 DVLRRPSKDEPLFAARVVYDLLFFFIVIIIVLNLIFGVIIDTFADLRSEK 109  -IV
                              M8

2600 QKKEEILKTTCFICGLERDKFDNKTVTFEEHIKEEHNMWHYLCFIVLVKV 2649 -I
 379 QKKEEILKTTCFICGLERDKFDNKTVSFEEHIKSEHNMWPYLYFIVLVKV 428  -II
 118 QKKEEILKTTCFICGLERDKFDNKTVSFEEHIKLEHNMWNYLYFIVLVRV 167  -III
 110 QKKEEILKTTCFICGLERDKFDNKTVSFEEHIKSEHNMWHYLYFIVLVKV 159  -IV

2650 KDSTEYTGPESYVAEMIRERNLDWFLRMRAMSLVSSDSEGEQNELRNLQE 2699 -I
 429 KDPTEYTGPESYVAQMITEKNLDWFPRMRAMSLVSNEGDSEQNEIRNLQE 478  -II
 168 KNKTDYTGPESYVAQMIKNKNLDWFPRM     LGSTS               200  -III
 160 KDPTEYTGPESYVAQMI                                   176  -IV

2700 KLESTMKLVTNLSGQLSELKDQMTEQRKQKQRIGLLGHPPHMNVNPQQPA 2749 -I
 479 KLESTMSLVKQLSGQLAELKEQMTEQRKNKQRLGFLGSNTP HVNHHMPP 527  -II


     *                                                   2750 -I
     H*                                                  529  -II
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FIG. 1. Sequences of IP_3R-II, -III, and -IV compared with a portion of the original P400 IP_3R of Furuichi *et al.* (12) (IP_3R-I). The single-letter amino acid code is used. Amino acids identical in the IP_3R-I and any of the three other sequences are highlighted in boldface type. Putative membrane-spanning regions M1 through M8 (14) are underlined. Stars indicate stop codons.

the brain we observed labeling selectively in cerebellar Purkinje cells (Fig. 5). We failed to detect clear labeling in any other brain region, though the PCR data suggest that IP_3R-IV is expressed in the forebrain as well.

DISCUSSION

In this study we present evidence for three IP_3R-related sequences that, taken with the previously known IP_3R-I, suggests the existence of IP_3Rs derived from three or four distinct genes. Because of close similarities between IP_3R-II and IP_3R-IV, it is possible that they reflect alternatively spliced forms of a single gene and not distinct gene products. Arguing against this possibility is the PCR analysis using primers for divergent untranslated regions, which suggests that IP_3R-IV is more abundant than IP_3R-II. Our IP_3R-II and IP_3R-III appear to be the mouse versions of the rat IP_3R-II and human IP_3R-III cloned by Südhof *et al.* (19). Südhof *et al.* (19) showed that expression of the N-terminal portion of IP_3R-II yielded specific IP_3 binding, indicating that it is a physiological IP_3R and suggesting that the other isoforms are also functional.

Comparison of sequences for the four receptors is difficult, as full-length sequences have been obtained only for IP_3R-I and IP_3R-II. Our sequences for mouse IP_3R-II, IP_3R-III, and IP_3R-IV are largely from the C terminus. We observe a high degree of homology among these three receptor forms in the membrane-spanning domains and C terminus. The most

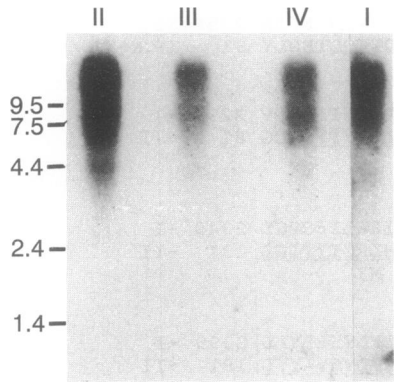

FIG. 2. Northern blot analysis of IP_3R-II (lane II), IP_3R-III (lane III), IP_3R-IV (lane IV), and IP_3R-I (lane I). Size markers (kilobases) refer to single-stranded RNAs. Total RNA was prepared from mouse placenta at day 19 of gestation. Similar quantities were loaded in each lane. However, the specific activities of the probes were not necessarily equivalent, so that relative levels of expression for the different IP_3Rs cannot be deduced from this figure. On shorter exposure, IP_3R-II and IP_3R-I appeared to have two bands, around 10 and 10.5 kilobases.

striking divergence is apparent in the cytoplasmic loop between membrane-spanning domains M6 and M7, suggesting that this portion of the receptor is important for functions that differentiate the various IP_3Rs.

Tissue localizations observed by PCR blot analysis and *in situ* hybridization suggest distinct functions for the various receptors. IP_3R-III appears to be most highly concentrated in smooth muscle layers of the intestine. In this tissue, and

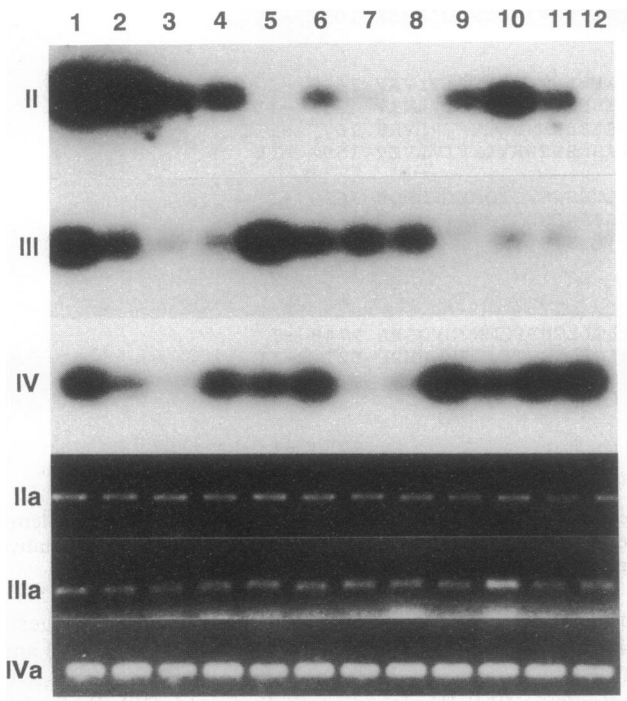

FIG. 3. PCR analysis of tissue expression of the different IP_3Rs. First-strand cDNA was transcribed from total RNA by using oligo(dT) as a primer. For each experiment actin cDNA and the specific IP_3R cDNA were coamplified in the same tube, and quantities of template were adjusted so that actin cDNA was amplified approximately equally in each tube. Panels: II, IP_3R-II; III, IP_3R-III; IV, IP_3R-IV; IIa, actin control for IP_3R-II (ethidium bromide-stained); IIIa, actin control for IP_3R-III; IVa, actin control for IP_3R-IV. Lanes: 1, brain; 2, lung; 3, liver; 4, kidney; 5, gastrointestinal tract; 6, testes; 7, thymus; 8, spleen; 9, spinal cord; 10, placenta; 11, cerebellum; 12, forebrain.

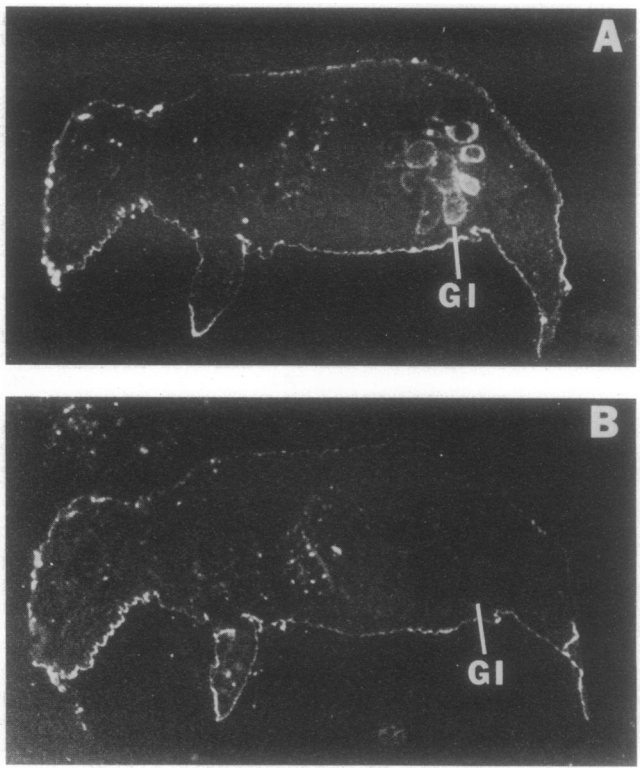

FIG. 4. *In situ* hybridization analysis of IP_3R-III expression in sagittal fetal mouse sections (day 20 of gestation). Probes were transcribed from the IP_3R-III insert-containing clone by using T3 and T7 RNA polymerases to yield antisense and sense probes with the same specific activity. (*A*) Antisense probe. (*B*) Sense probe. GI, gastrointestinal tract.

perhaps in other smooth muscles, levels of mRNA for IP_3R-III may approach or exceed those of IP_3R-I. The IP_3R system is the principal determinant of the influence of neurotransmitters and hormones upon gastrointestinal motility mediated by Ca^{2+} release. IP_3R-III may be important for these actions.

The thymus, enriched in T lymphocytes, displays appreciable substantial levels of IP_3R-II and -III. In attempts to clone IP_3Rs from thymus libraries, we have isolated several cDNA clones that appear to represent IP_3R-II or IP_3R-IV (data not shown). A plasma membrane form of IP_3R has been

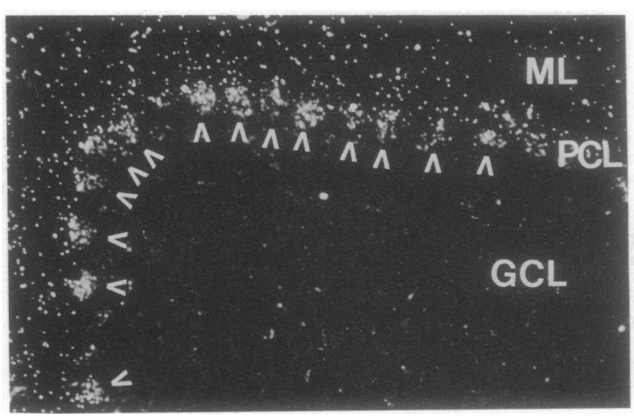

FIG. 5. *In situ* hybridization analysis of IP_3R-IV expression in Purkinje cells of the cerebellum. Probes were two antisense oligonucleotides directed against the area of high divergence in the region between membrane-spanning domains M6 and M7. Sense-orientation probes gave no specific hybridization. Arrowheads mark silver grains over Purkinje cells. ML, molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer.

identified by electrophysiological (7) and biochemical (25) means in T lymphocytes. IP_3R associated with lymphocyte plasma membranes differs from the receptor associated with endoplasmic reticulum in inositol phosphate specificity, heparin sensitivity, and the selective association of sialic acid with the plasma membrane receptor (25). IP_3R-II or IP_3R-IV could represent this distinct receptor isoform.

In the cerebellum, both IP_3R-I and IP_3R-IV are expressed in Purkinje cells and IP_3R-III may also occur there. Thus, at least two and perhaps three IP_3Rs occur in Purkinje cells. How might they relate functionally? In Purkinje cells, IP_3Rs can be localized immunocytochemically to several different structures, including smooth endoplasmic reticulum, rough endoplasmic reticulum, hypolemmal cisternae, and especially cisternal stacks of smooth endoplasmic reticulum (4–6, 26). Recent immunocytochemical studies have localized in Purkinje cells an additional endoplasmic reticulum subcompartment enriched in Ca^{2+}-binding proteins that may correspond to the calcisomes of other cells (27). Immunocytochemical studies at the electron microscopic level might permit differential localizations of the subtypes of IP_3R in Purkinje cells. Alternatively, different forms of the receptor may occur together in the same structures. The IP_3R tetramer could comprise subunits from different isoforms of IP_3R, a pattern that is well established for many neurotransmitter-gated channels (28–30) and that might contribute to cooperative Ca^{2+} release and multiple conductance states (31–33).

We thank Shihua Li, Audrey Bentley, and Sylvia Gärtner for expert technical assistance. We thank Irmi Sures, Andreas Dreps, and Lu Mostav for many helpful discussions. This work was supported by Public Health Service Grant MH18501 and Research Scientist Award DA-00074 to S.H.S., Grant MH43040 and a National Association for Research on Schizophrenia and Depression (NARSAD) Young Investigator Award to C.A.R., Training Grant GM07309 to S.K.D., and a gift from Bristol-Myers Squibb. C.A.R. is a Pew Scholar in the Biomedical Sciences.

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