Tissue-specific alternative splicing of mouse brain type ryanodine receptor/calcium release channel mRNA

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Received 6 August 1996

Abstract **We detected alternative splicing of the mouse brain type ryanodine receptor (RyR3) mRNA. The splicing variant was located in the transmembrane segment. The non-splicing type (RyR3-II) included a stretch of 341 bp, and that of the 13th codon was stop codon TAA. Reverse transcription-polymerase chain reaction (RT-PCR) analysis shows that RyR3-II mRNA was expressed in various peripheral tissues and brain at all developmental stages. However, interestingly, the splicing type (RyR3-I) mRNA was detected only in the cerebrum. These findings suggest that the splicing variants RyR3-I and RyR3-II may generate functional differences of RyR3 in a tissue-specific manner.**

Key words: Ryanodine receptor; Calcium release channel; Mouse brain; Alternative splicing; RT-PCR

1. Introduction

The ryanodine receptor (RyR) is one of the major Ca^{2+} release channels, which is activated by Ca^{2+}, causing Ca^{2+}-induced Ca^{2+} release (CICR) from intracellular Ca^{2+} stores [1–3]. RyR proteins are formed from 550–565 kDa monomers, and to date three isoforms are known to be encoded by different genes: the skeletal muscle type (RyR1) [4], the cardiac type (RyR2) [5,6], and the brain type (RyR3) [7,8]. RyRs are expressed in many tissues, but their expression patterns are different [8–11]. Early studies revealed the localization of RyRs: RyR1 primarily to skeletal muscle, RyR2 to heart and brain, and RyR3 to brain and some peripheral tissues. However, recent studies involving RNase protection analysis or reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed that all the RyRs are widely expressed in brain and peripheral tissues; RyR1 is also expressed in cerebellar Purkinje cells, RyR2 is the major isoform in almost all parts of the central nervous system, and RyR3 is expressed in some restricted areas of the brain, especially the corpus striatus, thalamus and hippocampus [9–13].

The alternative splicing of mRNAs of the RyRs genes may possibly generate further heterogeneity rather than the above three types. In the case of IP_3R, its subtypes are expressed in a tissue-specific or developmental manner due to various alternative splicing of mRNAs in the N-terminal and modulatory regions [14,15]. Indeed, alternative splicing of RyR1 and RyR2 mRNAs has also been reported [5,16]. However, no alternative splicing of RyR3 mRNA has been reported. In the present study, we detected alternative splicing of mouse RyR3 mRNA which was localized in the transmembrane segment. We also investigated the distribution of the splicing pattern in various tissues and at various developmental stages by means of reverse transcription-polymerase chain reaction (RT-PCR) analysis. The results show that the splicing pattern was in a tissue-specific manner.

2. Materials and methods

2.1. Cloning and sequencing of mouse cDNAs for the splicing (RyR3-I) and non-splicing (RyR3-II) types of RyR3

A newborn BALB/c mouse brain cDNA library in phage λgt11 (Stratagene) was used for PCR amplification of the transmembrane segment in the mouse RyR3 gene. The oligonucleotide primers for PCR were designed in the previous study, as follows: sense primer 3A, 5′-TTCATCCTGCTGTTTTATAAGGT-3′, and antisense primer 3B, 5′-CAGATGAAGCATTTGGTCTCCAT-3′; corresponding to nucleotide numbers 13724–13746 and 14860–14882 of human RyR1, respectively [4,17]. The buffer for PCR comprised 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.25 mM each dCTP, dGTP, dTTP and dATP, 80 nM oligonucleotide primers, 2 units of Ampli-Taq polymerase (Perkin Elmer), and 5 μl of cDNA, in a final volume 50 μl. The samples were subjected to 5 cycles of 1 min at 94°C, 1 min at 37°C, and 2 min at 72°C, and then 40 cycles of 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C, with a final extension step at 72°C for 7 min. The PCR product was resolved by electrophoresis on a 2% agarose gel, and 1.5 kbp, 1.2 kbp and 1156 bp fragments were visualized by staining with ethidium bromide. Then the PCR products were immediately subcloned into TA plasmids (Invitrogen), and both strands of the cloned PCR products containing the three types of fragments were sequenced with a model 377A DNA Sequencer (ABI) according to the manufacturer's instructions. The sequence analysis showed that the 1156 bp fragment was mouse RyR3 cDNA, the 1.2 kbp fragment mouse RyR2 cDNA, and the 1.5 kbp fragment mouse RyR3 cDNA with an insertion of 341 bp [10]. Furthermore, a new pair of oligonucleotide primers was designed to sequence the 614 bp fragment including the 341 bp insertion, as follows: sense primer SP1F, 5′-AGGTGATCAACAAGTATGGA-3′, and antisense primer SP1R, 5′-CAACAGATGAGCAGCAAAGA-3′. We repeated the cloning and sequencing steps more than five times to rule out sequencing errors due to PCR amplification.

2.2. Sequencing of genomic DNA around the splicing site

To determine the sequence of genomic DNA around the splicing site, genomic DNA from adult BALB/c mouse liver was purified with a RapidPrep Genomic DNA Isolation Kit for Cells and Tissues (Pharmacia Biotech) according to the manufacturer's instructions. The PCR amplification of the genomic DNA was carried out through

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Abbreviations: RyRs, ryanodine receptors; RT-PCR, reverse transcription-polymerase chain reaction; IP_3R, inositol 1,4,5-triphosphate receptor; CICR, Ca^{2+}-induced Ca^{2+} release; E, embryonic day; NB, newborn

The nucleotide sequences reported in this paper have been submitted to the GenBank®/EMBL/DDBJ Data Bank with accession numbers D85590 and D85591.

PII S0014-5793(96)01022-8

42 cycles of 1 min at 94°C, 2 min at 45°C, and 2 min at 72°C, with a final extension step at 72°C for 7 min, oligonucleotides SP1F and SP1R being used as PCR primers. The 940 bp fragment of the PCR product was subcloned into plasmids and sequenced according to the same methods as above.

2.3. RT-PCR assay for expression of RyR3-I and RyR3-II

BALB/c mice, from embryos to adults (embryonic day 15 (E15), newborn (NB), 1, 2, 3, 8, and 15 weeks) were prepared for analysis of the expression level of RyR3-I and RyR3-II mRNAs in the brain, and 20-day-old BALB/c mice were prepared for distribution analysis of RyR3-I and RyR3-II in some tissues (cerebrum, cerebellum, skeletal muscles, heart, lung, liver, spleen, kidney and peripheral blood). mRNAs from mouse tissues were purified with a Quick-Prep Micro mRNA Purification Kit (Pharmacia Biotech), and each purified mRNA was converted into cDNA with a Ready-To-Go T-Primed First-Strand Kit (Pharmacia Biothech) according to the manufacturer's instructions. The PCR amplification of cDNA was carried out through 30 cycles of 1 min at 94°C, 2 min at 43°C, and 2 min at 72°C, with a final extension step at 72°C for 7 min, oligonucleotides SP1F and SP1R being used as PCR primers. β-Actin cDNA of brains at all stages was amplified as a control to determine the quantities of cDNA under the same conditions as described above. The PCR products were electrophoresed on 2% agarose gels and then transferred to Hybond N+ membranes (Amersham). The 100 base oligonucleotide probes used were designed by the sequence of mouse RyR3 cDNA upstream of the splicing site, as follows: SP2, GATCTCTATGGAG-CAGAGCGCATCGCTGAACTCCTGGGTTT GGACAAAAATG-CCCTTGACTTTAGCCCAGTAGAAGAGGCCA AAGCAGAGG-CAGCATCTC. The probe was labelled by the 3′-end labelling method with [γ-^{32}P]ATP, using a Megalabel Kit (TaKaRa). Hybridization was performed in a hybridization buffer comprising 10×Denhardt's solution, 6×SSC, 0.1% SDS, and 50 μg/ml salmon sperm DNA overnight at 65°C. Next, the membranes were washed with 6×SSC at room temperature, and 2×SSC and 0.5×SSC at 65°C (1×SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Autoradiography was then performed. Densitometric analysis of signals was performed with a BAS-1000 (Fuji Film).

3. Results

3.1. Sequence of the 341 bp insertion of RyR3-II

By sequence analysis of the 1156 bp and 1.5 kbp fragments, which were PCR amplified using primers 3A and 3B, we identified a splicing variant of mouse RyR3 cDNA. In addition, a new pair of oligonucleotide primers SP1F and SP1R were designed to sequence around the splicing site. The inclusion and exclusion of 341 bp generated two variants RyR3-I and RyR3-II, respectively (Fig. 1A). The 341 bp splicing site was located between M2 and M3 in the transmembrane segment of the RyR3 gene. The 13th codon of inclusion site was coded stop codon TAA, and the sequences on the 5′ and 3′ sides were GT and AG, respectively (Fig. 1B).

We next analysed the genomic organization around the splicing site by PCR amplification of the mouse RyR3 genomic DNA with primers SP1F and SP1R, and a 940 bp fragment was amplified and sequenced. Comparison of the sequences of the cDNA and genomic DNA revealed that the 940 bp genomic DNA fragment between SP1F and SP1R contains three exons and two introns, 341 and 326 bp, respectively. The sequence of the 341 bp intron was the same as that of the splicing region, and that of the 326 bp intron was also in accordance with the GT/AG rule.

3.2. Expression of RyR3-I and RyR3-II in the brain at various developmental stages

To determine the expression level of RyR3-I and RyR3-II mRNAs in the brain at various developmental stages (from E15 to 15 weeks), the RT-PCR assay with primers SP1F and SP1R was utilized, following Southern hybridization analysis. The 100 base probe for Southern hybridization was designed so as to be common to RyR3-I and RyR3-II, being designed from the sequence of upstream of the splicing site. The lengths of signals were expected to be 273 bp for RyR3-I and 614 bp for the RyR3-II. The results showed that RyR3-I and RyR3-II mRNAs were expressed in the whole brain at all stages. Densitometric analysis revealed the expression of RyR3-II mRNA was less than that of RyR3-I at all stages (Fig. 2A).

3.3. Expression of RyR3-I and RyR3-II in a tissue-specific manner

To determine the tissue distribution of RyR3-I and RyR3-II, the RT-PCR assay was utilized by the same method as described above (Fig. 2B). As a result, 614 bp signals of RyR3-II were detected in cerebrum, cerebellum, skeletal muscle, heart, liver, spleen, kidney and peripheral blood, but not lung, the signal in the pancreas was being weaker than in the other tissues. To our surprise, 273 bp signals, which represent RyR3-I, were not detected in any tissues except cerebrum.

4. Discussion

RyR is one of the Ca^{2+} release channels from intracellular stores in skeletal and cardiac muscles, and functions as CICR [18–23]. To date, three isoforms are known to be encoded by different genes [4–8,24]. Alternative splicing of mRNAs could also generate subtypes rather than the three RyR isoforms in a tissue-specific or developmental manner. Indeed, alternative splicing of the RyR1 and RyR2 genes has already been demonstrated [5,15,16,25]. In the present study, we detected alternative splicing of mouse RyR3 mRNA. RyR3-II included stop codon TAA in the transmembrane domain between M2 and M3. Furthermore, the splicing variant was in a tissue-specific manner.

The sequences of RyR3 and the other RyRs exhibit 70% homology [8,26], and alignment of the amino acid sequences revealed significant similarity between RyR and IP_3R [8,27–29]. It has also been reported that there are four hydrophobic segments (M1–M4) in the carboxy-terminal region of RyR3. Since, in particular, the M3 and M4 segments show remarkable amino acid sequence similarity to the corresponding region of IP_3R, this region may contribute to the Ca^{2+} release mechanism which is common to both intracellular Ca^{2+} release channels [8]. The four highly hydrophobic transmembrane segments in RyR3-I can not be conserved in RyR3-II because of a stop codon between M2 and M3. These findings suggest that the function of RyR3-II as a Ca^{2+} release channel may be different from that of RyR3-I and the other RyRs.

Some agents are known that affect RyR, such as ryanodine, caffeine, ruthenium red, cyclic adenosine diphosphoribose, a metabolite of nicotinamide adenine dinucleotide [30–33]. However, the sensitivity to ryanodine or caffeine of RyR3 is known to differ from that of RyR1 or RyR2, and vice versa. Giannini et al. [7] demonstrated that RyR3 in mink lung epithelial cells was insensitive to caffeine. Hakamata et al. [8] reported that RyR3 was insensitive to caffeine but sensitive to ryanodine. A recent study involving myocytes from dyspedic mice lacking RyR1 revealed that RyR3 functions as a CICR channel sensitive to caffeine and ryanodine, but RyR3 had the lowest Ca^{2+} sensitivity among the members

of the mammalian RyR family [34]. These findings suggest that differences in the functioning of RyR3 as a Ca^{2+} release channel or the sensitivity to some agents may be due to a structural difference between RyR3 and the other RyRs. Also, RyR3-II which has an incomplete channel region, may contribute to the lower function of RyR3 as a Ca^{2+} release channel.

RyR3 is known to be widely expressed not only in the central nervous system but also in peripheral tissues such as mammalian skeletal muscle, heart, lung, stomach, spleen, pancreas, intestine, kidney, and human Jurkat T-cells [8,10,11,17]. We also determined the tissue distributions of RyR3-II and RyR3-I mRNAs by RT-PCR assay, the RyR3-II mRNA being detected in the cerebrum, cerebellum, skeletal muscle,

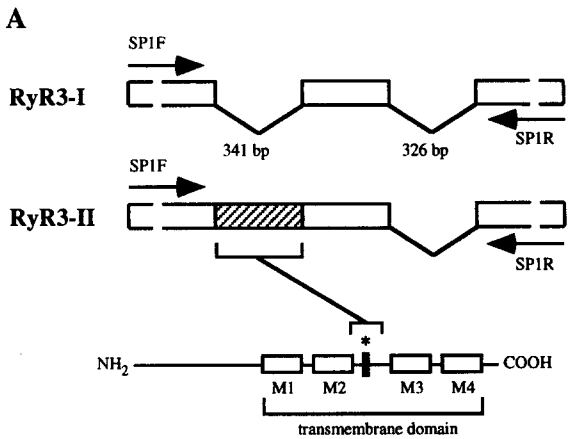

Fig. 1. (A) Schematic diagram of mouse RyR3 showing the location of the alternative splicing site, and the splice variants RyR3-I and RyR3-II. The alternatively spliced region was located between M2 and M3 in the putative transmembrane segment of the RyR3 gene, and the inclusion and exclusion of a stretch of 341 bp generated two variants RyR3-II and RyR3-I. The expected sizes of the PCR products are 273 bp for RyR3-I and 614 bp for RyR3-II by PCR using primers (SP1F and SP1R), respectively. Exons and the 341 bp inclusion of RyR3-II are shown by boxes and shaded box. (B) The partial sequences of the nucleotide (top) and the deduced amino acid (in single-letter code; bottom) of mouse RyR3-II cDNA. The sequence of the 341 bp inclusion site (enclosed with solid lines) is the same as that of the 341 bp intron, and is in accordance with the GT/AG rule. The 13th codon is a stop codon TAA (asterisk). The intron-exon boundary where interrupted by the 326 bp intron in genomic DNA is shown by a triangle.

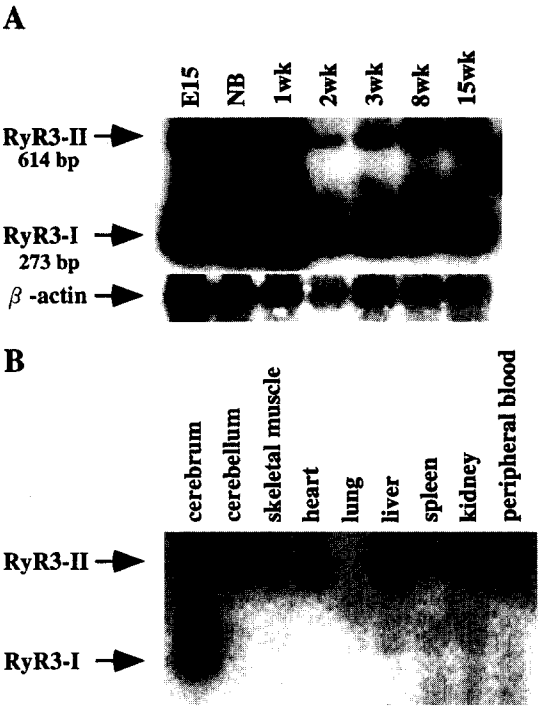

Fig. 2. Autoradiograms after blot hybridization analysis of RT-PCR products from mouse brain, from E15 to 15 weeks (A), and peripheral tissues (B). RyR3 cDNA from brains at several developmental stages (E15–15 weeks) and peripheral tissues were amplified with primers SP1F and SP1R. The signals of PCR products of 614 and 273 bp, which correspond to RyR3-II and RyR3-I, respectively, are indicated. The signal of β-actin at each developmental stage is indicated at the bottom.

heart, liver, spleen, kidney and peripheral blood. However, to our surprise, RyR3-I mRNA was only detected in the cerebrum. These findings suggest that these splicing variants occur in a tissue-specific manner; RyR3-II may be a superior type in peripheral tissues, RyR3-I may be a neuronal type of RyR3, and the mechanism of Ca^{2+} release via RyR3 may differ between brain and peripheral tissues. In addition, the coexistence of the two splicing variants of RyR3 mRNA in the brain suggests that RyR-I and RyR3-II might interact and contribute to regulate intracellular Ca^{2+} release in neurons.

The densitometric analysis indicated that the expression of RyR3-II mRNA was much less than that of RyR3-I. Although the ryanodine-binding study indicated that a ryanodine-binding site was present in liver microsomes, and ryanodine binding to the liver endoplasmic reticulum was inhibited by caffeine [35], RyR3 mRNA was not detected in liver on RT-PCR [10,11], which might be due to the low level of expression of the RyR3 gene and protein in the liver, as suggested by Giannini et al. [10]. Moreover, despite the fact that RyR3 mRNA was detected in the skeletal and cardiac muscles, the RyR3 protein was not detected in microsomes from these tissues by Western blot analysis [10]. However, in our study, only RyR3-II mRNA was detected in peripheral tissues including skeletal and cardiac muscles and liver, RyR3-I mRNA not being detected. Therefore, previous studies might have failed to detect RyR3 mRNA in the liver due to the low amount of RyR3-II mRNA. Furthermore, differences in the high-affinity binding of ryanodine between liver microsomes and sarcoplasmic reticulum [35,36] may be due to the fact that

RyR1, RyR2 and RyR3-II are expressed in skeletal muscle but only RyR3-II is expressed in the liver.

In the present study, we have demonstrated tissue-specific alternative splicing of mouse RyR3 mRNA for the first time. In addition, this variant splicing may generate differences in its function as a Ca^{2+} release channel, and the coexistence of the two splicing variants in the cerebrum may contribute to the regulation of the intracellular Ca^{2+} release in the central nervous system.

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