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

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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-I) 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-II) mRNA was detected only in the cerebral. 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 Ca2+ release channels, which is activated by Ca2+. causing Ca2+ induced Ca2+ release (CICR) from intracellular Ca2+ 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 RNA 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 cardiobellus 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 callosum, thalamus and hippocampus [9-13].

The alternative splicing of mRNAs of the RyR genes may possibly generate further heterogeneity rather than the above three types. In the case of IP3R, 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 various 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'-CAGATGAAGCATTTGGTCTCCAT-3'; corresponding to nucleotide numbers 1279-1261 and 1480-1462 of human RyR1, respectively [4,14]. The primer for PCR comprised 50 nM KCL 10 mM Tris-HCl, pH 5.3, 0.25 mM each dCTP, dGTP, dTTP and dATP, 80 nM of each oligonucleotide primers, 2 units of Ampli-Taq polymerase (Perkin Elmer), and 5 μl of cDNA, in a final volume of 50 μl. The samples were subjected to 5 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C, and then 40 cycles of 1 min at 94°C, 2 min at 56°C, and 2 min at 72°C, with a final extension step at 72°C for 5 min, then PCR products were purified by electrophoresis on a 2% agarose gel, and 1.5 kb, 1.2 kb and 1.15 kb fragments were visualized by autoradiography. The PCR products were immediately subcloned into TA plasmids (Invitrogen), and both strands of the cloned PCR products covering the three types of segments were sequenced with a model 377A DNA Sequencer (ABI) according to the manufacturer's instructions. The sequence analysis showed that the 1.15 kb fragment was mouse RyR3 cDNA, the 1.2 kb fragment covered mouse RyR2 cDNA, and the 1.35 kb fragment covered mouse RyR3 cDNA with an insertion of 141 bp [9]. Furthermore, a new pair of oligonucleotide primers was used to sequence the 641 bp fragment inducing the 341 bp insertion, as follows: sense primer SP1F, 5'-AGGTGATCAACAAGTATGGA-Y, and antisense primer SP1R, 5'-CAACAGATGAGCAGCAAAGAG-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 hearts was purified with a RNase-Free Micro Universal 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
42 up + 1 min at 72°C, 2 min at 45°C, and 5 min at 4°C with a final extension step at 72°C for 2 min. oligonucleotide primers SPI and SPII were used as PCR primers. The 940 bp fragment of the PCR product was subcloned into pBluescript II and sequenced according to the manufacturer's instructions. The PCR amplification of cDNA was carried out for 30 cycles of 1 min at 94°C, 2 min at 45°C, and 5 min at 72°C with a final extension step at 72°C for 2 min. oligonucleotides SP1F and SP1R were designed to sequence around the splicing site. The inclusion segments (M1-M4) in the carboxy-terminal region of RyR3. The sequence of the 341 bp intron was the same as that of the known 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 tissue distribution of RyR3-I and RyR3-II, the RT-PCR assay was utilized by the same method as described above (Fig. 2A). As a result, 614 bp signals of RyR3-I 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-II, were not detected in any tissues except cerebrum.

3.3. Discussion

RyR is one of the Ca2+ release channels from intracellular stores in skeletal and cardiac muscles and functions as a CICR mechanism [8-23]. To date, three isoforms have been identified by different genes [4-8,24]. Alternative splicing of mRNA 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 [5,12,16,25]. In the present study, we detected alternative splicing of mouse 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 IP3R [8-27-29]. It has also been reported that there are four hydrophilic segments (M1-M4) in the cathode-terminal region of RyR3. Since, in particular, the M3 and M4 segments show remarkable amino acid sequence similarity in the corresponding region of IP3R, this region may contribute to the Ca2+ release mechanism which is common to both intracellular Ca2+ release channels [8]. The four highly hydrophilic transmembrane segments in RyR3-I cannot 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 Ca2+ release channel may be different from that of RyR3-I and the other RyRs.

Some agents known to affect RyR, such as ryanodine, caffeine, forskolin, calcium influx, and cyclic adenosine monophosphate, a metabolite of nicotinamide adenine dinucleotide [30-33]. However, the sensitivity to ryanodine or caffeine of RyR3 is different from that of RyR1 or RyR2, and vice versa. Ginnai et al. [7] demonstrated that RyR3 in mink lung epithelial cells is insensitive to caffeine. Hakomura et al. [8] reported that RyR3 was insensitive to caffeine but sensitive to ryanodine. A recent study involving myocytes from dysgenic mice lacking RyR1 revealed that RyR3 functions as a CICR channel sensitive to caffeine and ryanodine, but RyR3 had the lowest Ca2+ 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. A
also, RyR3-II which has an incomplete domain, 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]. 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 cerebral, cerebellum, skeletal muscle,
heart, liver, spleen, kidney, and peripheral blood. However, to
our surprise, RyR3-I mRNA was only detected in the cere-
brum. 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 be-
tween brain and peripheral tissues. In addition, the coex-
istence of the two splicing variants of RyR3 mRNA in the
brain suggests that RyR3-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.

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-II. 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. Boxes 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, and sarcoplasmic
reticulum [35,36] may be due to the fact that

Fig. 2. Autoradiograms after blot hybridization analysis of RT-PCR
products from mouse brain, from E15 to 15 weeks (A), and periph-
erial tissues (B). BcDNA 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 \(\beta\)-actin at each developmental stage is indi-
cated at the bottom.
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 Ca²⁺ release channel, and the coexistence of the two splicing variants in the cerebrum may contribute to the regulation of the intracellular Ca²⁺ release in the central nervous system.

References