Smooth Muscle Tissues Express a Major Dominant Negative Splice Variant of the Type 3 Ca\(^{2+}\) Release Channel (Ryanodine Receptor)*

Received for publication, October 10, 2002, and in revised form, November 25, 2002
Published, JBC Papers in Press, December 5, 2002, DOI 10.1074/jbc.M210410200

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It is well known that the type 3 Ca\(^{2+}\) release channel (ryanodine receptor, RyR3) exhibits strikingly different pharmacological and functional properties depending on the tissues in which it resides. To investigate the molecular basis for this tissue-dependent heterogeneity, we examined the primary structure of RyR3 from various tissues by reverse transcription polymerase chain reaction and DNA sequence analysis. As many as seven alternatively spliced variants of RyR3 were detected. Ribonuclease protection assays revealed that one of these splice variants, RyR3 (AS-8a), which lacks a 29-amino acid fragment (His\(^{4400}\)-Lys\(^{4434}\)) encompassing a predicted transmembrane helix, was highly expressed in smooth muscle tissues, but not in skeletal muscle, the heart, or the brain. Although the RyR3 (AS-8a) splice variant lacks a functional Ca\(^{2+}\) release channel when expressed alone in HEK293 cells, it was able to form functional heteromeric channels with reduced caffeine sensitivity when co-expressed with the wild type RyR3. Interestingly, this RyR3 splice variant was also able to form heteromeric channels with and suppress the activity of the type 2 ryanodine receptor (RyR2). Tissue-specific expression of RyR3 splice variants is therefore likely to account for some of the pharmacological and functional heterogeneities of RyR3. These observations also reveal a novel mechanism by which a splice variant of one RyR isoform (RyR3) can suppress the activity of another RyR isoform (RyR2) via a dominant negative effect.

Ryanodine receptors (RyRs)† were initially described in the sarcoplasmic reticulum of striated muscles. It is now known that there are three mammalian RyR isoforms (RyR1, RyR2, and RyR3) and that they are expressed in a variety of cells and tissues (1–3). RyR1 is predominantly expressed in skeletal muscle, whereas RyR2 is mainly expressed in heart, brain, and smooth muscles. The expression of RyR3 has been detected in a broad range of tissues, including brain, smooth muscles, and skeletal muscle (4). RyR1 and RyR2 function as Ca\(^{2+}\) release channels and play an essential role in excitation-contraction coupling in striated muscles (5–8). However, the physiological role and channel properties of RyR3 remain elusive.

Depending on the tissues in which it is expressed, RyR3 exhibits different functional properties. In skeletal muscle, it functions as a caffeine- and ryanodine-sensitive Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel similar to RyR1 and RyR2 (9) and is involved in amplifying the Ca\(^{2+}\) signals generated by RyR1 (10). On the contrary, in smooth muscle cells, RyR3 forms a ryanodine-sensitive but caffeine-insensitive Ca\(^{2+}\) release channel (11, 12) and may negatively regulate the activity of RyR2 and/or RyR1 (13). The expression of RyR3 has also been demonstrated in human Jurkat T-lymphocytes and mink lung epithelial cells. Interestingly, these cells also exhibit ryanodine-sensitive, caffeine-insensitive Ca\(^{2+}\) release activity (14, 15). These observations have led to the notion that RyR3 expressed in smooth muscles and peripheral tissues possesses unique functional properties, although the molecular mechanism underlying this tissue-specific function of RyR3 is unknown.

To account for the pharmacological and functional heterogeneity of RyR3, it has been proposed that smooth muscle and peripheral tissues may express a unique isoform of RyR3 as a result of alternative splicing (16, 17). To test this hypothesis, we systematically investigated the existence of alternatively spliced variants of RyR3 expressed in the uterus by amplifying and sequencing the entire ~15-kb coding region of RyR3. Our results show that RyR3 expressed in smooth muscle tissues is extensively modified by alternative splicing and that one of the splice variants, RyR3 (AS-8a), is highly and selectively expressed in smooth muscle tissues. Functional characterization reveals that this major RyR3 splice variant does not form a functional channel when expressed alone but is able to form functional heteromeric channels with the wild type RyR3 and RyR2. Our data provide the molecular basis for the tissue-dependent heterogeneity of RyR3 and demonstrate for the first time that a splice variant of RyR3 is able to interact with and suppress the activity of another RyR isoform, RyR2, via the formation of heteromeric channel complexes.

MATERIALS AND METHODS

Amplification of RyR3 cDNA by RT-PCR—Total RNA was isolated from various rabbit tissues as described previously (18). Briefly, first strand cDNA was prepared from total RNA using the SuperScript Preamplification System (Invitrogen). The entire 15-kb coding region of RyR3 was amplified by PCR using Taq DNA polymerase and eight pairs of primers (CP-1F/CP-1R through CP-8F/CP-8R) (17). The following PCR primers were used to amplify the splice regions: AS2-F, 5′-CCA-TCTCCTTCCGGCATC-3′; AS2-R, 5′-TTACGGCCGCGGTCTC-3′; AS5-F, 5′-TGAAGAGTGTGGAC-3′; AS5-R, 5′-ACCCACTTTCCGGATG-3′; AS6-F, 5′-ACAACTCTTTCCGGAT-3′; AS6-R, 5′-CCCGTCCGGTCTGCT-3′; AS7-F, 5′-ATGGAGGCAACGCCGGT-3′; AS8-F, 5′-GGGTGGAATATCATC-3′; AS8a-R, 5′-AGCTCCCTTCCCGGCA-3′; AS8b-F, 5′-GTCACTGAAATGGAC-3′; and AS8b-R, 5′-AGCTGATCTTCATGATG-3′.

Ribonuclease Protection Assay—A ribonuclease protection assay was performed as described previously (18). The human 5′ untranslated region of RyR3 mRNA was synthesized by in vitro transcription using T7 RNA polymerase. The ribonuclease protection assay was performed as described previously (18).

* This work was supported by research grants from the Canadian Institutes of Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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This paper is available on line at http://www.jbc.org
carried out using the RPA II kit from Ambion according to the manufacturer's instructions. Briefly, a RyR3 DNA fragment (231 bp) comprising the AS-8a splice region (87 bp), a 114-bp 5′-flanking, and a 30-bp 3′-flanking sequence was generated by PCR and cloned into pBluescript. The plasmid was linearized and used as template to synthesize [32P]-labeled RNA probe using the T7 RNA polymerase. The [32P]-labeled RNA probe was hybridized with total RNA isolated from various tissues followed by treatment with RNases. The protected probes were analyzed by polyacrylamide gel electrophoresis and autoradiography.

Site-directed Mutagenesis and RNA Transfection—Construction of the full-length RyR3 cDNA and insertion of the c-Myc epitope tag into RyR3 after glutamate 4318 have been described previously (17, 19). The HEK293 cells (ATCC, Rockville, MD) were transfected with either the vector or the in-frame insertions of full-length cDNA were either excluded or included (Fig. 1B and C). The nature of this heterogeneity was further investigated by DNA sequence analysis. Two or three clones of each PCR fragment representing each unique digestion pattern were sequenced. This analysis revealed that as many as seven regions in RyR3 cDNA were either excluded or included (Fig. 1B and Table I). The exclusion of regions AS-5, 6, 7, 8a, and 8b led to deletions of 5, 6, 29, and 51 amino acids, respectively, without changing the reading frame. The exclusion of region AS-7 also led to a substitution of Arg for Gly3724. On the other hand, exclusion of regions AS-2 and AS-5 resulted in a frameshift. Exclusion of region AS-4 represented an insertion of a single serine residue after valine 2271 as a result of using an 18-bp insertion in the 3′-flanking region.

**RESULTS**

**Identification of Alternately Spliced Variants of RyR3**—The entire coding region of RyR3 from rabbit uterus was amplified by using RT-PCR and eight pairs of PCR primers (Fig. 1A). The resulting overlapping cDNA fragments (PCR1 through PCR8) were subcloned into the pBluescript vector. At least 14 individual clones of each RT-PCR fragment were isolated and analyzed by restriction endonuclease digestion. A single digestion pattern was observed when individual clones of the PCR1, PCR3, and PCR4 fragments were digested with multiple restriction endonucleases (data not shown). On the other hand, two or more digestion patterns were detected in clones of PCR2, PCR5, PCR6, PCR7, and PCR8, indicating that each of these later PCR products contain heterogeneous DNA species.

The nature of this heterogeneity was further investigated by DNA sequence analysis. Two or three clones of each PCR fragment representing each unique digestion pattern were sequenced. This analysis revealed that as many as seven regions in RyR3 cDNA were either excluded or included (Fig. 1B and Table I). The exclusion of regions AS-6, AS-7, AS-8a, and AS-8b led to deletions of 5, 6, 29, and 51 amino acids, respectively, without changing the reading frame. The exclusion of region AS-7 also led to a substitution of Arg for Gly1724. On the other hand, exclusion of regions AS-2 and AS-5 resulted in a frameshift. Different from other regions, AS-4 represented an insertion of a single serine residue after valine 2271 as a result of using an alternative splicing acceptor site (Table I). A comparison between the intron/exon boundaries of the human RyR3 gene and human codons.
The sequences of these deleted regions (Table I) reveal that each of these regions corresponds to one or more exons of the human RyR3 gene. Because the complete intron/exon boundaries of the rabbit RyR3 gene are unknown, each spliced exon is indicated by the corresponding exon number of the human RyR3 gene. Horizontal lines represent splicing that yields the wt RyR3 cDNA, whereas tilted lines represent alternative splicing found in this study in which one or two exons (boxed nucleotide sequences) were excluded, with the exception of AS-4. In the case of AS-4, sequence analysis of the rabbit RyR3 genomic DNA around the AS-4 region revealed two potential acceptor sites, cag, in the intron sequence (italics), leading to two alternatively spliced transcripts with or without the insertion of three nucleotides encoding the amino acid, serine. In the cases of AS-2, AS-5, AS-6, or AS-8a, one entire exon was spliced out. In the case of AS-7, alternative splicing resulted in a deletion of an exon and the change of glycine at position 3724 to arginine. The location and length of the deleted cDNA sequences corresponding to one or two exons are indicated.

| Name | Location and Length | Alternative Splice Junction | Type |
|------|---------------------|-----------------------------|------|
| AS-2 | Δ2359-2575 (217 bp) | GTC AA GTC AGT CAG GGT GTT | frameshift |
| AS-4 | +3 bp at 6812       | CCG GAA GTT aagctgat ototag cag | in-frame |
| AS-5 | Δ8714-8763 (50 bp)  | GCC AGC C TG TTC TTT TTC GGC AGC | frameshift |
| AS-6 | Δ10021-10035 (15 bp)| TCA AAA GCC ATG GTC TAA AAG TCT GGA | in-frame |
| AS-7 | Δ11153-11170 (18 bp)| GGA ACA GTC ATT CGT GGT GAA AAG | in-frame |
| AS-8a| Δ13216-13302 (87 bp)| TTA CGC CA CAC TAC TAT AAG GTC ACA | in-frame |
| AS-8b| Δ14371-14523 (153 bp)| ATG GAG ACT AAA C TAC CTG G TGC TTT CAG GAA TTC | in-frame |

The AS-8a Splice Variant Is Highly and Selectively Expressed in Smooth Muscle Tissues—Of all splice variants detected in smooth muscle tissues, splice variant AS-8a (containing deletion of the AS-8a region) appears to be highly expressed (Fig. 2e). To further quantify the level of the AS-8a splice variant, we performed a ribonuclease protection assay using total RNA isolated from uterus, aorta, heart, brain, and diaphragm and an antisense3P-labeled RNA probe that encompasses the AS-8a region. A major band with a size of ~114 bases and a minor band of ~231 bases were detected in uterus and aorta (Fig. 3). The 231-base band corresponds to transcripts that contain the AS-8a region (AS-8a ()), whereas the 114-base band represents transcripts that lack the AS-8a region (AS-8a (−)). The relative level of the 114-base AS-8a (−) band, as determined by phosphorimaging analysis, was more than 5-fold of that of the 231-base AS-8a (+) band in uterus and aorta. In contrast, the relative level of AS-8a (−) band (231 bases) detected in heart, brain, and diaphragm was about 5-fold of that of the AS-8a (+) band in uterus and aorta. Hence, consistent with the results of RT-PCR (Fig. 2), these ribonuclease protection assay results indicate that the AS-8a splice variant is highly expressed in smooth muscle tissues but not in the heart, brain, or diaphragm.

Formation of RyR3 (AS-8a)/RyR3 (wt) Heteromeric Complexes—To investigate the functional properties of the highly expressed AS-8a splice variant, we constructed a RyR3 cDNA
containing a deletion of the 87-bp AS-8a region and expressed the RyR3 (AS-8a) cDNA in HEK293 cells. Functional characterization revealed that the AS-8a splice variant did not form a functional Ca\(^{2+}\) release channel when expressed alone in HEK293 cells.

![Image 326x512 to 554x737]

**Fig. 2.** Tissue distribution of RyR3 splice variants. The presence of alternatively spliced RyR3 variants, AS-2 (a), AS-5 (b), AS-6 (c), AS-7 (d), AS-8a (e), and AS-8b (f), expressed in various rabbit tissues was assessed by RT-PCR. PCR products were electrophoresed in 5% polyacrylamide gels, stained with ethidium bromide, and visualized under UV light. Plasmid clones containing RT-PCR fragments (PCR2 through PCR6) isolated from uterus were used as controls. Control 1 (lane 1) shows PCR products using a plasmid clone that contains one of the splice regions as the template, whereas control 2 (lane 2) shows PCR products using a plasmid clone that lacks one of the splice regions as the template. Control 1 for AS-6 (c) is not available, because RyR3 expressed in uterus does not contain the AS-6 region. No signals were detected in RT-PCR in the absence of reverse transcriptase. The sizes of PCR fragments that contain (+) or lack (−) the corresponding splice region are indicated on the right.

**Fig. 3.** Splice variant RyR3 (AS-8a) is highly expressed in smooth muscle tissues. The relative level of the AS-8a splice variant expressed in various tissues was examined by ribonuclease protection assay. An antisense \(^3\)P-labeled RNA probe (307 bases) was hybridized with total RNA isolated from uterus, aorta, heart, brain, and diaphragm. The probes protected from RNases digestion were analyzed by polyacrylamide gel electrophoresis and autoradiography. The 231-base fragment represents RyR3 transcripts lacking the AS-8a region, whereas the 114-base fragment represents RyR3 transcripts lacking the AS-8a region.

**Fig. 4.** Splice variant RyR3 (AS-8a) is capable of forming heteromeric channel complexes with RyR3 (wt). Immunoprecipitation was carried out with anti-c-Myc (a) or anti-HA (b) antibodies using cell lysates prepared from HEK293 cells transfected with RyR3 (wt, c-Myc) (12 µg), RyR3 (AS-8a, HA) (12 µg), or RyR3 (wt, c-Myc) (6 µg) plus RyR3 (AS-8a, HA) (6 µg). The immunoprecipitates (IP) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with the anti-RyR (34C), anti-HA, or anti-c-Myc antibodies as indicated. WB, Western blot.

Because both the alternatively spliced and unspliced forms of AS-8a transcripts are present in smooth muscle tissues, it is possible that the AS-8a splice variant and the unspliced RyR3 form heteromeric RyR3 channels. To test this possibility, we inserted the c-Myc antibody epitope tag into RyR3 (wt) and the HA tag into RyR3 (AS-8a) and co-expressed them in HEK293 cells. Interactions between RyR3 (wt, c-Myc) and RyR3 (AS-8a, HA) were examined by immunoprecipitation. Fig. 4a (top panel) shows that the anti-c-Myc antibody was able to pull down RyR3 (wt, c-Myc) from lysate of HEK293 cells transfected with RyR3 (wt, c-Myc) or co-transfected with RyR3 (wt, c-Myc) and RyR3 (AS-8a, HA) (lanes 1 and 3) but not RyR3 (AS-8a, HA) from cells transfected with RyR3 (AS-8a, HA) alone (lane 2). The same anti-c-Myc immunoprecipitates were subsequently blotted with the anti-HA antibody. This antibody recognized a major band in the anti-c-Myc immunoprecipitate from HEK293 cells co-transfected with RyR3 (wt, c-Myc) and RyR3 (AS-8a, HA) (lane 1) but not from cells transfected with RyR3 (wt, c-Myc) alone (lane 1, bottom panel). These data indicate that the anti-c-Myc and anti-HA antibodies are specific and that RyR3 (AS-8a, HA) when expressed together with RyR3 (wt, c-Myc) can be co-precipitated with RyR3 (wt, c-Myc) by the anti-c-Myc antibody.

We also performed the reciprocal experiment in which the RyR3 (AS-8a, HA) was immunoprecipitated by the anti-HA antibody and the presence of RyR3 (wt, c-Myc) in the anti-HA immunoprecipitates was detected by Western blotting. As shown in Fig. 4b (top panel), the anti-HA antibody pulled down the RyR3 (AS-8a, HA) (lanes 2 and 3) but not the RyR3 (wt, c-Myc) (lane 1). The anti-c-Myc antibody did not cross-react with the RyR3 (AS-8a, HA) (lane 2, bottom panel), but recognized a major band in the anti-HA immunoprecipitate from cells co-transfected with RyR3 (AS-8a, HA) and RyR3 (wt, c-Myc) (lane 3, bottom panel). Hence, although the anti-HA antibody is unable to pull down RyR3 (wt, c-Myc), it is able to precipitate the RyR3 (wt, c-Myc) in the presence of RyR3 (AS-8a, HA). Taken together, these results demonstrate that the
RyR3 (AS-8a) splice variant is able to form heteromeric complexes with the wild type RyR3.

The RyR3 (AS-8a) Splice Variant Is Able to Form Functional Heteromeric Channels with a RyR3 Mutant—The ability of the RyR3 (AS-8a) splice variant to form heteromeric complexes was further assessed by complementation analysis using a RyR3 mutant. The rationale being that if RyR3 (AS-8a) is able to form heteromeric channels with other RyR3 variants, co-expression of RyR3 (AS-8a) with a nonfunctional RyR3 mutant may produce a functional heteromeric channel, because two nonfunctional mutants may complement each other’s defects. During deletion analysis of RyR3, we generated a caffeine-insensitive RyR3 mutant, RyR3 (ΔE2256-T2429). To test whether RyR3 (AS-8a) can complement this mutant, HEK293 cells were transfected individually or in combination with RyR3 (AS-8a) and RyR3 (ΔE2256-T2429). As indicated in Fig. 5, HEK293 cells transfected with RyR3 (AS-8a; Fig. 5a) or RyR3 (ΔE2256-T2429; Fig. 5b) alone did not respond to caffeine, whereas co-expression of RyR3 (AS-8a) and RyR3 (ΔE2256-T2429) led to a caffeine-sensitive Ca\(^{2+}\) release (Fig. 5, c–e). These data suggest that the RyR3 (AS-8a) splice variant is able to form functional heteromeric channels with other RyR3 variants.

Co-expression of RyR3 (AS-8a) Decreases the Sensitivity of RyR3 (wt) to Caffeine Activation—The functional aspect of the RyR3 (AS-8a)/RyR3 (wt) heteromeric complexes was further investigated by examining their caffeine response. HEK293 cells were co-transfected with RyR3 (wt) and pcDNA3 vector cDNA or with RyR3 (wt) and RyR3 (AS-8a). The peaks of Ca\(^{2+}\) release from aliquots of transfected cells induced by different concentrations of caffeine (0.05–20 mM) were measured. As shown in Fig. 6, Ca\(^{2+}\) release from RyR3 (wt)-transfected cells was activated by caffeine with an EC\(_{50}\) of 0.87 ± 0.09 mM (mean ± S.E., n = 3). On the other hand, Ca\(^{2+}\) release from cells co-transfected with RyR3 (wt) and RyR3 (AS-8a) was activated by caffeine with an EC\(_{50}\) of 2.4 ± 0.02 mM (n = 3). Therefore, co-expression of RyR3 (AS-8a) reduces the caffeine sensitivity of RyR3 (wt), suggesting that by forming heteromeric channels, the RyR3 (AS-8a) splice variant can influence the activity of RyR3 (wt).

The RyR3 (AS-8a) Splice Variant Is Capable of Interacting with and Suppressing the Activity of RyR2—We have recently shown that RyR3 (wt) is capable of forming heteromeric channels with RyR2 (wt) in HEK293 cells (24). One would expect that the RyR3 (AS-8a) splice variant would be also able to form heteromeric channels with RyR2 (wt). To directly test this hypothesis, we co-expressed HA-tagged RyR3 (AS-8a, HA) with c-Myc-tagged RyR2 (wt, c-Myc) in HEK293 cells. The association of RyR3 (AS-8a, HA) with RyR2 (wt, c-Myc) was examined by immunoprecipitation followed by immunoblotting. As indicated in Fig. 7A (panel a), in addition to precipitating RyR2 (wt, c-Myc) from cells transfected with RyR2 (wt, c-Myc) alone (lane 1, top panel) or co-transfected with RyR3 (AS-8a, HA) and RyR2 (wt, c-Myc) (lane 3, top panel), the anti-c-Myc antibody was also able to precipitate RyR3 (AS-8a, HA) from cells co-transfected with RyR3 (AS-8a, HA) and RyR2 (wt, c-Myc) (lane 3, bottom panel) but not from cells transfected with RyR3 (AS-8a, HA) alone (lane 2). Similarly, in a reciprocal experiment, the anti-HA antibody was able to pull down RyR2 (wt, c-Myc) from cells co-transfected with RyR3 (AS-8a, HA) and RyR2 (wt, c-Myc) (Fig. 7A, panel b, lane 3), but not from cells transfected with RyR2 (wt, c-Myc) alone (Fig. 7A, panel b, lane 1). Taken together, these data indicate that RyR3 (AS-8a, HA) and RyR2 (wt, c-Myc) when expressed together can be co-precipitated either by anti-HA or anti-c-Myc antibody, demonstrating that the RyR3 (AS-8a) splice variant, like RyR3 (wt), can form heteromeric complexes with RyR2 (wt).

To examine whether the physical interaction between RyR3 (AS-8a) and RyR2 (wt) affect the activity of RyR2 (wt), we co-transfected HEK293 cells with RyR2 (wt) and RyR3 (AS-8a) or RyR2 (wt) with vector DNA, pcDNA3. \(^{3}H\)Ryanodine binding to lysates of co-transfected HEK293 cells were determined. As shown in Fig. 7B, no \(^{3}H\)ryanodine binding was detected in cells transfected with RyR3 (AS-8a) alone, consistent with the results of Ca\(^{2+}\) release experiments (Fig. 5a) that showed that RyR3 (AS-8a) does not form a functional Ca\(^{2+}\) release channel when expressed alone in HEK293 cells. Furthermore, co-expression of RyR3 (AS-8a) reduced \(^{3}H\)ryanodine binding to RyR2 (wt) by about 50%. \(^{3}H\)ryanodine binding has been widely used as a functional assay for RyR channel activities, because ryanodine binds to only the open state of the channel. Thus, these studies indicate that the RyR3 (AS-8a) splice variant is able to suppress not only the activity of RyR3 (wt) (Fig.
RyR2 can be inhibited by a RyR3 splice variant through a heteromeric channels with reduced caffeine sensitivity (Figs. 5–7). Together, these observations reveal a novel heteromeric channel complexes with RyR2. Thus, it appears that the main functional role of RyR3 in smooth muscle cells differs heterogeneous in both function and regulation (3). One of its major heterogeneities is its response to caffeine activation. RyR3 expressed in skeletal muscle and the brain is caffeine- and ryanodine-sensitive (9, 16), whereas RyR3 expressed in uterine smooth muscle cells is ryanodine-sensitive but caffeine-insensitive (11, 12). The molecular basis for this tissue- or cell-dependent RyR3 heterogeneity is unclear. Our findings that the RyR3 (AS-8a) splice variant is highly expressed in smooth muscle tissues but not in skeletal muscle and the brain and that co-expression of this major splice variant with the wild type RyR3 reduces the caffeine sensitivity of RyR3 strongly suggest that tissue-specific expression of RyR3 splice variants, in particular the RyR3 (AS-8a) splice variant, may account for the heterogeneous caffeine response of RyR3 observed in different tissues or cells. In support of this view, the corresponding AS-8a region was found to be deleted in RyR3 from mink lung epithelial cells, which also display ryanodine-sensitive but caffeine-insensitive Ca$^{2+}$ release (14). Ryanodine-sensitive but caffeine-insensitive Ca$^{2+}$ release activity was also observed in human Jurkat T-cells (15), and it will be interesting to see whether the RyR3 (AS-8a) splice variant is expressed in these cells.

The physiological function of the AS-8a splice variant is unknown. Because caffeine activates RyR by sensitizing the channel to Ca$^{2+}$ activation, altered caffeine response may reflect changes in Ca$^{2+}$ regulation. In this context, it is of interest to know that RyR3 expressed in nonpregnant myometrial smooth muscle cells does not respond to activation by Ca$^{2+}$ and caffeine under normal sarcoplasmic reticulum Ca$^{2+}$ loading but becomes active when sarcoplasmic reticulum Ca$^{2+}$ loading is increased (27). This observation suggests that RyR3 from these smooth muscle cells has a reduced sensitivity to activation by luminal Ca$^{2+}$ and that its activity is normally suppressed. It remains to be explored whether the RyR3 (AS-8a) splice variant is highly expressed in these smooth muscle cells and whether this splice variant is involved in luminal Ca$^{2+}$ regulation.

Another observation that may provide some clues to the physiological role of the RyR3 (AS-8a) splice variant comes from a study using RyR3 knock-out mice. Cerebral artery smooth muscle cells isolated from these mice displayed an increased frequency of Ca$^{2+}$ sparks and spontaneous transient outward currents and a reduced myogenic tone as compared with the wild type cells (13). These findings led to the suggestion that Ca$^{2+}$ sparks and spontaneous transient outward currents in arterial vascular smooth muscle cells are negatively regulated by RyR3. The molecular mechanism by which RyR3 inhibits Ca$^{2+}$ spark frequency is not clear. It has been proposed that RyR1 and RyR2, but not RyR3, are responsible for Ca$^{2+}$ spark generation and that inhibition of Ca$^{2+}$ spark frequency may result from RyR3-mediated prolonged Ca$^{2+}$ release, which may then inactivate RyR1 or RyR2 (13). The findings that RyR3 expressed in myometrial smooth muscle cells is inactive under normal sarcoplasmic reticulum Ca$^{2+}$ loading and that the RyR3 (AS-8a) splice variant, the major form of RyR3 expressed in various smooth muscle tissues, does not form a functional Ca$^{2+}$ release channel when expressed alone in HEK293 cells is inconsistent with the idea of RyR3-mediated prolonged Ca$^{2+}$ release. Alternatively, our observation that the RyR3 (AS-8a) splice variant is able to interact physically with and suppress the activity of RyR2 suggests the interesting possibility that RyR3 expressed in smooth muscle cells as a splice variant may suppress Ca$^{2+}$ sparks, spontaneous transient outward currents, and myogenic tone by forming heteromeric channel complexes with RyR2. Thus, it appears that the main functional role of RyR3 in smooth muscle cells differs...
from that of RyR3 in skeletal muscle and the brain. Unlike RyR3 expressed in skeletal muscle, where it functions as a Ca\(^{2+}\) release channel, RyR3 expressed in smooth muscles or epithelial cells in the form of alternatively spliced variants may function largely as a suppressor of Ca\(^{2+}\) release.

The observation that very low levels of RyR3 transcripts that contain the AS-8a region were detected in various smooth muscle tissues as compared with those of the AS-8a splice transcripts is in agreement with this view (Fig. 2). An excess amount of the AS-8a splice variant would ensure that all of the wt RyR3 subunits would be oligomerized with the AS-8a, so that no homomeric wt RyR3 channels or heteromeric wt RyR3/AS-8a channels with high wt RyR3:AS-8a subunit ratios would be formed to suppress the activity of the wt RyR3 channels. Because AS-8a can also form heteromeric channels with wt RyR2, the AS-8a splice variant may also be involved in oligomerization with wt RyR2 where they are co-expressed, as shown in vascular smooth muscle. It is important to note that an excess amount of AS-8a does not completely suppress the activity of either wt RyR3 or wt RyR2. We were able to detect the activity of RyR3 or RyR2, although at reduced levels, after co-expression of AS-8a with wt RyR3 in a ratio of 15:1 (AS-8a:wt RyR3) or with RyR2 in a 10:1 ratio (AS-8a:wt RyR2) in HEK293 cells (Figs. 6 and 7).

In addition to AS-8a, several other splice regions in RyR3 have also been detected in various smooth muscle tissues (Fig. 2). Of these splice regions, AS-2 and AS-5 are of interest. They are located near the 5′-end and in the middle of the RyR3 cDNA, respectively (Fig. 1). Exclusion of each of these regions leads to a frameshift and is predicted to result in the synthesis of truncated RyR3 proteins containing the first NH\(_2\)-terminal \(-800\) and \(-2900\) amino acid residues, respectively. These truncated RyR3 proteins lack the COOH-terminal pore-forming region and, if expressed alone, would be nonfunctional. However, whether they can form heteromeric channel complexes with and thereby affect the channel activity of the full-length RyR3 or RyR2 remains to be assessed. We have previously shown that co-expression of NH\(_2\)-terminal fragments of RyR2 with overlapping COOH-terminal fragments produces functional Ca\(^{2+}\) release channels in HEK293 cells (28). This observation indicates that the NH\(_2\)-terminal region of RyR2 is able to interact functionally with the COOH-terminal region. It will be of interest to determine whether the NH\(_2\)-terminal regions of RyR3, corresponding to the AS-2 and AS-5 splice variants, are capable of interacting functionally with the COOH-terminal regions of RyR3 and RyR2.

The presence of these frameshifted splice variants raises the possibility that splice variants such as AS-8a, whose splice regions are located downstream of the frameshifted splice regions, are not made into proteins in smooth muscles, despite their existence at the RNA level. Based on the results shown in Fig. 2, it is clear that not all of the RyR3 transcripts are alternatively spliced in the AS-2 or AS-5 regions, because con-