Generation and Characterization of Mutant Mice Lacking Ryanodine Receptor Type 3*

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The ryanodine receptor type 3 (RyR-3) functions as a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) channel and is distributed in a wide variety of cell types including skeletal muscle and smooth muscle cells, neurons, and certain non-excitable cells. However, the physiological roles of RyR-3 are totally unclear. To gain an insight into the function of RyR-3 in vivo, we have generated mice lacking RyR-3 by means of the gene targeting technique. The mutant mice thus obtained showed apparently normal growth and reproduction. Although Ca\(^{2+}\)-induced Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores of the mutant skeletal muscle differed in Ca\(^{2+}\) sensitivity from that of wild-type muscle, excitation-contraction coupling of the mutant muscle seemed to be normal. Moreover, we could not find any significant disturbance in the smooth muscle and lymphocytes from the mutant mice. On the other hand, the mutant mice showed increased locomotor activity, which was about 2-fold greater than that of the control mice. These results indicate that the loss of RyR-3 causes no gross abnormalities and suggest that the lack of RyR-3-mediated Ca\(^{2+}\) signaling results in abnormalities of certain neurons in the central nervous system.

Intracellular Ca\(^{2+}\) stores play a critical role in the regulation of the cytoplasmic Ca\(^{2+}\) concentration, and the ryanodine receptor (RyR) \(^3\) mediates Ca\(^{2+}\) release from the stores as a major class of Ca\(^{2+}\) release channels (1, 2). RyR was first identified as the Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) channel in the sarcoplasmic reticulum (SR) of skeletal muscle, and it has been demonstrated that the channel activity is enhanced by increased cytosolic Ca\(^{2+}\) concentrations (3). The purified RyR protein was shown to form a homotetramer with the characteristic "foot" structure, which spans the gap between the SR and transverse tubules (4). Furthermore, it was deduced by cloning the cDNA that the RyR is composed of ~5,000 amino acid residues with the carboxyl-terminal channel region containing transmembrane segments and the remaining large cytoplasmic portion constituting the "foot" structure (5).

Through DNA cloning studies, we now know that mammalian tissues have at least three subtypes of RyR, namely the skeletal muscle (RyR-1), the cardiac (RyR-2), and the brain (RyR-3) subtypes, that are derived from distinct genes (6, 7). Functional properties of RyR-1 and RyR-2 have been studied extensively because these two receptors are present predominantly in skeletal muscle and cardiac muscle, respectively. RyR-1 is essential for excitation-contraction (E-C) coupling of skeletal muscle (8) and is thought to function as the Ca\(^{2+}\) release channel linked mechanically with the cell surface voltage sensor (2, 3). In cardiac muscle RyR-2 is thought to mediate Ca\(^{2+}\) release triggered by Ca\(^{2+}\) influx through the voltage-gated Ca\(^{2+}\) channel (2, 3). On the other hand, the physiological functions of RyR-3 are unknown.

The complete structure of RyR-3 was first determined in rabbit brain (9), and the existence of the corresponding molecule has been established in several animal species by cDNA cloning studies (10–14). RyR-3 has been reported to exist in a wide variety of cell types: neurons, smooth muscle cells, skeletal muscle cells, lymphocytes, and certain non-excitable cells (9–16). We have previously demonstrated, using skeletal muscle from mutant mice lacking RyR-1, that RyR-3 functions as a CICR channel with lower Ca\(^{2+}\) sensitivity than that of other subtypes (12). However, the biological roles of RyR-3-mediated Ca\(^{2+}\) signaling have not yet been elucidated. To study roles of RyR-3 in the whole-animal level, we generated mutant mice lacking RyR-3. This paper examines our first characterization of the mutant mice.

**Experimental Procedures**

Generation of Mice Lacking RyR-3—A randomly primed mouse brain cDNA library was screened with the cDNA insert from clone pRR705 (5) as a probe to yield λMBRR224 carrying a 5′-terminal cDNA segment for the mouse RyR-3. Then, a mouse genomic library was screened with the 75-base pair EcoRI/Neol fragment derived from λMBRR224 to yield λMBRGS1 carrying exon 1 in the mouse RyR-3 gene and with the 0.6-kilobase EcoRI/EcoRV fragment to yield several clones, including λMBRRG14, carrying exon 2. Targeting vectors were constructed using the genomic segments thus obtained; in pBBP1–12 the ~0.9-kilobase Smal/BamHI fragment containing the putative promoter region and exon 1 was replaced by the neomycin resistance gene from pMC1neo poly(A) (Stragagene), and in pBBPP2–11 termination codons followed by the neomycin resistance gene were inserted at the StyI site in exon

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2 (see Fig. 1). The RyR-3 gene targeting and generation of knockouts using 1 embryonic stem (ES) cells (17) were performed essentially as described previously (8). We have established three lines of mice carrying targeted mutations in the RyR-3 gene, those derived from ES clones numbered 205, 653, and 621; clone 205 was isolated from cells transfected with pBRRP1-12, and clones 653 and 621 were isolated from pBRRP2-11-transfected cells.

Blot Analysis—For Southern blot analysis (18), DNA samples from ES clones or tail biopsies were digested with restriction enzymes. Resulting DNA fragments were electrophoresed on 1% agarose gels and transferred to Durapore membranes (Stratagene). Genomic DNA fragments were labeled with [α-32P]dCTP (19) and used as hybridization probes.

Subtype-specific antibody to RyR-3 and brain microsomes were prepared according to previously described methods (16). The microsomal proteins were electrophoresed on an SDS-5% polyacrylamide gel and analyzed as described previously (8).

Analysis of Muscles—Muscle fiber bundles dissected from limbs of newborn mice were tied with silk filaments at both ends and mounted between a pair of stainless steel rods, one of which was attached to a force transducer (AE801, Aker, Norway). The muscle preparation was immersed in PSS containing (in mM): 150 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 5 HEPES, 5.6 glucose (pH 7.4). Supramaximal field electrical stimulation (20 V, 0.5-ms duration) was delivered by a pair of platinum wires placed on both sides of the preparation.

Skinned fibers were prepared from a small bundle of muscle fibers that had been treated with saponin (50 μg/ml) for 60 min in a relaxing solution containing (in mM): 4.76 ATP, 5.54 MgMS, 108.6 KMS, 20 NaCl, 20 PIPES, 1 EGTA (pH 7.0). The microfluorometric method for the measurement of CICR in skinned fibers has been described previously (20).

Flow Cytometric Analysis and Lymphocyte Proliferation—Surface markers of the isolated cells from spleen, bone marrow, and thymus were analyzed on an Epics Profile flow cytometer (Coulter Electronics). The reagents used for direct staining were anti-CD4 and -B220 phycoerythrin-conjugated monoclonal antibodies and anti-CD8 and -Thy1.2 fluorescein isothiocyanate-conjugated monoclonal antibodies (Pharminagen). In each cell preparation 104 viable cells were analyzed; dead cells were excluded from the analysis using forward and side scatter parameters.

The proliferative response of spleen cells was measured by [3H]thymidine incorporation (21). Cultures of spleen cells (each 2 × 104 cells/0.1 ml) were stimulated with either concanavalin A (5 μg/ml, Sigma), lipopolysaccharides (20 μg/ml, Sigma), or mouse interleukin-2 (500 units/ml, Genzyme) for 3 days and pulsed with 1 μCi of [3H]thymidine (1.29 TBq/mmol, ICN Biomedicals Inc.) for the final 24 h of incubation. The cells were harvested, and the incorporated radioactivity was measured.

Measurement of Locomotor Activity—Locomotor activity experiments were conducted as described previously (22). Each mouse (8–9-week-old male) was placed in a round testing cage (18 cm in diameter and 18 cm high) of tilting-type ambulometer, which was supported at the center of its bottom and three points around it (MA001, O'Hara & Co., Ltd., Japan), and allowed to explore for 3 h. On the following day, locomotor activity was measured in the identical test, and the data were recorded as six blocks of 10-min intervals.

RESULTS AND DISCUSSION

Disruption of RyR-3 Gene—We have produced two independent lines of mutant mice, in that the RyR-3 gene has been disrupted at either exon 1 or exon 2 (Fig. 1, A and D). Both strains of mice with the mutant genes showed the expected pattern of gene disruption as identified by Southern blotting analysis (Fig. 1, B and E). By crosses between heterozygous mutants, homozygotes were yielded in distribution following Mendelian rules; hence they seemed to survive fetal development normally. The homozygous mutants that grew and reproduced normally without propensity to premature death were indistinguishable in appearance from wild-type and the heterozygous littersmates. However, immunoblot experiments showed that there is no detectable RyR-3 protein in the brain from the homozygous mutants (Fig. 1, C and F). Thus, it is likely that both mutations are null mutations of RyR-3. These observations indicate that loss of RyR-3 causes no apparent abnormalities in mice. Two lines of RyR-3-deficient mice, de-
The FK-506 binding protein with a relative molecular mass of 12 kDa, a soluble receptor for the immunosuppressant drug FK-506, is tightly associated with RyR and may function as an endogenous modulator for CICR channels (25, 26). Furthermore, cultured Jurkat T-lymphocytes express only RyR-3 among the RyR subtypes, and ryanodine as an activator of RyR channels affects the physiological functions of smooth muscle. This is supported by the fact that the mutant mice seem to possess no significant defects in their digestive or circulation systems.

Lymphocytes from RyR-3-deficient Mice—The expression of the RyR-3 gene is also known in smooth muscle tissues (9). The Ca$^{2+}$ sensitivity of CICR in the longitudinal smooth muscle of guinea pig colon is quite similar to that in muscle lacking RyR-1 and expressing solely RyR-3 (12, 20). Therefore, it may be possible that RyR-3 is a major component of CICR channels in smooth muscle and takes part in the physiological contraction. However, we could not find any difference in caffeine- and noradrenaline-induced contractions between arterial smooth muscles from the RyR-3-deficient and wild-type mice (data not shown). Thus, it is likely that the loss of RyR-3 does not affect the physiological functions of smooth muscle. This is supported by the fact that the mutant mice seem to possess no significant defects in their digestive or circulation systems.

In flow cytometric analysis using antibodies to specific cell surface markers (see “Experimental Procedures”), we identified no abnormal populations of T- or B-lymphocytes in spleen,
and control mice, we could not detect any difference in \(^{3}H\)thymidine uptake under non-stimulating or stimulating conditions with the mitogens tested. These results indicate that RyR-3 is not essential for maturation or growth of lymphocytes and do not suggest the involvement of Ca\(^{2+}\) mobilization via RyR-3 in lymphocyte proliferation.

**Locomotion Activity in RyR-3-deficient Mice**—Expression of RyR-3 in the brain (9, 15, 16) indicates a functional contribution of RyR-3 channels in Ca\(^{2+}\) signaling of neurons in the central nervous system. To survey behavioral abnormalities, we first examined the locomotor activity of the mutant mice because the homozygous mutants seemed to be restless in comparison with the control littermates. As shown in Fig. 4, the homozygous mutants exhibited about two times greater locomotor activity than the heterozygous mutants and wild-type mice (p < 0.05; two-way analysis of variance). Locomotion activities of all types of mice decreased within a habituation period of approximately 40 min; however, during the final period of this test the RyR-3-deficient mice were still more active than the control mice. Thus, we conclude that the congenital lack of RyR-3 results in enhanced levels of basal locomotor activity.

Although locomotor activity is controlled by complex neural circuits in the brain interconnecting the sensory and motor systems, the main control centers for the activity include the frontoparietal cortex, basal ganglia, and thalamus where RyR-3 expression is shown to be predominant (15, 16). Abnormal Ca\(^{2+}\) signaling of certain neurons in these regions caused by a lack of RyR-3 may result in the locomotor hyperactivity. Another important center modulating movement resides in the cerebellum. Since the level of RyR-3 expression is low in cerebellum and the RyR-3-deficient mice did not manifest any ataxic behavior, the cerebellar system may not play a crucial role in the observed hyperactivity. Other experiments are needed to examine further behavioral abnormalities in the mutant mice.

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