A Region of the Ryanodine Receptor Critical for Excitation-Contraction Coupling in Skeletal Muscle*

(Received for publication, January 21, 1997)

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Ca2⁺ release mediated by the ryanodine receptor (RyR) regulates many important cell functions including excitation-contraction (E-C) coupling in skeletal muscle, by which membrane depolarization controls the opening of RyR via the dihydropyridine receptor. Among the three RyR subtypes, RyR-1 mediates skeletal muscle E-C coupling, whereas RyR-2 and RyR-3 cannot substitute for RyR-1. We carried out expression experiments using cultured mutant skeletal myocytes not having intrinsic intracellular Ca2⁺ release channels to study the structure-function relationship of amino acid residues 1303–1406 in RyR-1 (D2 region). In this region the amino acid sequences are highly divergent between RyR-1 and RyR-2, and the corresponding sequence is lacking in RyR-3. Expression of RyR-1 but not of RyR-2 rescued E-C coupling in the mutant cells. Deletion of either the entire D2 region or its N-terminal half from RyR-1 preserved the function of RyR-1 as a Ca2⁺ release channel but resulted in the loss of E-C coupling. Substitution of the D2 region for the corresponding sequence of RyR-2 had no effect on the function of RyR-1. These results indicate that the presence of the D2 region is critical for E-C coupling in skeletal muscle, although the D2 region alone cannot determine the functional difference between RyR-1 and RyR-2.

The ryanodine receptors (RyRs) are a family of channels that mediate the release of intracellular Ca2⁺ stores and comprise three subtypes derived from distinct genes (1–3). The function of RyRs was first recognized and most extensively studied in skeletal muscle, although the study of RyRs has been expanded to cardiac and smooth muscle cells, nerve cells, and component the functions of which are still poorly understood. Indeed, targeted disruption of the gene encoding RyR-1 resulted in the loss of E-C coupling (10), whereas skeletal muscle cells in RyR-3-deficient mice show normal E-C coupling (11). Although all subtypes of RyR mediate Ca2⁺-induced Ca2⁺ release (CICR), neither RyR-2 nor RyR-3 is capable of substituting for RyR-1 in skeletal muscle E-C coupling (12, 13). Thus, the three subtypes of RyR have clear functional differences. Although the overall amino acid sequence identity among the RyR subtypes is 67–70% (14), there are several regions where the amino acid sequences significantly diverge. These regions are referred to as D1, D2, and D3 (15). It is an interesting possibility, therefore, that these divergence regions may correspond to the different functions of the RyR subtypes.

Among the divergence regions of the RyRs, the D2 region of RyR-1 (amino acid residues 1342–1403) is contained within the stretch of amino acid residues 1303–1406, the corresponding sequence of which is entirely absent in RyR-3 (14). In this study, we refer to the residues 1303–1406 of RyR-1 as the D2 region and examine its functional significance. First we cultured skeletal myocytes from mutant mice lacking both RyR-1 and RyR-3. The cells were then transfected with expression plasmids carrying mutated RyR-1 cDNAs with deletions or substituted with the region of RyR-2 corresponding to the D2 region to examine if the mutated RyR-1 can mediate E-C coupling. The results obtained indicate that the presence of the D2 region in RyR-1 is essential for E-C coupling in skeletal muscle but is not for the CICR per se.

EXPERIMENTAL PROCEDURES

Mutant Mice, Cell Culture, and Transfection Experiments—The method of generation of mice carrying the disrupted RyR-1 or RyR-3 gene is described elsewhere (10, 11). Neonates simultaneously homozygous for both mutations were obtained by mating double heterozygotes. The genotypes of all the neonates used in the present experiments were determined by polymerase chain reaction analysis (10, 11). Primary cultured myocytes were prepared from newborn mice, and expression plasmids were co-transfected with the green fluorescent protein (GFP) expression plasmid (Life Technologies, Inc.) using LipofectAMINE (Life Technologies, Inc.) as described previously (13). After 3–5 days of culture in differentiation medium, myocytes were used in the experiments. Successfully transfected myocytes were identified by the fluorescence of GFP.

Intracellular Ca2⁺ Measurements—Cultured cells were incubated in a physiological salt solution (13) containing 20 μM Fura-2 for 30–35 min. The fluorescence intensity of Ca2⁺ indicator-loaded cultured cells was imaged using a cooled CCD camera (Photometrics) attached to an inverted Olympus IX70 microscope with a 40× (UPlanApo) objective. The excitation wavelength was 470 nm for GFP and 340 nm for Fura-2. Two-dimensional images composed of 128 × 128 pixels were acquired every 0.2 or 0.5 s (13).

Construction of Expression Plasmids—The plasmid pCAGGS (16)
was digested with HindIII, blunted using Klenow fragment, and self-ligated to delete the HindIII site. The resulting plasmid was cleaved with EcoRI and ligated to a synthetic polylinker carrying EcoRI, XhoI, HindIII, NotI, and Nhel sites to generate the expression plasmid pCAGPL. The expression plasmid pYT-1 was constructed by inserting the 15-kilobase pair HindIII fragment from pRRL11 (17), containing the entire coding sequence of the rabbit RyR-1, into the HindIII site of pCAGPL in the same orientation with respect to the β-actin promoter. The plasmid pYT-2 was generated by deleting nucleotide residues 3907–4218 of the RyR-1 cDNA (see Ref. 18 for the residue numbers) from pYT-1; to generate the Apal site in pYT-2, a base conversion, T to A at position 4221, was introduced that causes no amino acid substitution. The plasmids pYT-3 and pYT-4 were generated by deleting residues 3907–4101 and 4069–4218 from pYT-1, respectively; base substitutions T to A at position 4104 in pYT-3, G to T at position 4068, and T to A at position 4221 in pYT-4 cause no amino acid changes. The plasmid pYT-5 was generated by inserting the fragment 3946–4200 of RyR-2 cDNA (see Ref. 19 for the residue numbers) into the Apal site of pYT-2.

RESULTS AND DISCUSSION

**Ca**<sup>2+</sup> Transients in Cultured Myocytes Lacking RyR-1 or RyR-3 or Both—Myocytes isolated from the skeletal muscle of wild-type, RyR-1-deficient, and RyR-3-deficient mice as well as from double mutant mice lacking both RyR-1 and RyR-3 were cultured. The cultured cells from the wild-type and RyR-3-deficient mice responded with an intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]) increase to electrical stimulation even in the absence of extracellular Ca<sup>2+</sup> (Fig. 1, A and B) in accordance with the results obtained in intact muscle bundles (11). However, electrical stimuli were unable to induce an increase in [Ca<sup>2+</sup>] i in RyR-1-deficient and double-mutant myocytes in the absence of extracellular Ca<sup>2+</sup> (not shown). These results confirm that RyR-1 is required for skeletal muscle E-C coupling.

Upon application of 25 mM caffeine, a [Ca<sup>2+</sup>] i increase was observed in control, RyR-1-deficient, and RyR-3-deficient myocytes (Fig. 1, A, B, and C). However, no caffeine-induced [Ca<sup>2+</sup>] i increase was observed in double-mutant myocytes (Fig. 1D). When cyclopiazonic acid, an inhibitor of SR Ca<sup>2+</sup>-ATPase (20), was applied to the double-mutant myocytes, a [Ca<sup>2+</sup>] i increase was observed in the absence of extracellular Ca<sup>2+</sup> (not shown). Therefore, even though Ca<sup>2+</sup> was stored in the SR, caffeine failed to induce Ca<sup>2+</sup> release in double-mutant myocytes. Caffeine has been shown to induce Ca<sup>2+</sup> release through activation of one of the RyR subtypes (12, 21). Hence, the results obtained indicate that no RyR is expressed in the double-mutant myocytes.

In our previous studies we showed that RyR-1-deficient myocytes responded with a slow [Ca<sup>2+</sup>] i increase of long duration to electrical stimulation in the presence of 10 mM Ca<sup>2+</sup> and 10 μM Bay K-8644, a Ca<sup>2+</sup>-channel agonist, in the extracellular solution (10, 12). However, it was not determined whether this [Ca<sup>2+</sup>] i increase was due solely to an influx of Ca<sup>2+</sup> or to CICR from the SR secondary to the Ca<sup>2+</sup> influx. Here we show that upon electrical stimulation, a [Ca<sup>2+</sup>] i increase that was similar in size and kinetics to that of RyR-1-deficient myocytes was induced in double-mutant myocytes in the presence of the high concentration of extracellular Ca<sup>2+</sup> and Bay K-8644 (Fig. 1, C and D). The peak height of the Fura-2 fluorescence intensity increase relative to the resting fluorescence and the time to reach 50% of the peak height were 1.13 ± 0.02 and 0.12 ± 0.12 s for RyR-1-deficient myocytes and 1.13 ± 0.02 and 0.39 ± 0.14 s for double-mutant myocytes (mean ± S.E., n = 8 for both cell types). There was no statistically significant difference in the [Ca<sup>2+</sup>] i between the two types of mutant myocytes (p > 0.3, t test). These observations suggest that the electrically evoked [Ca<sup>2+</sup>] i increase observed in RyR-1-deficient myocytes was due to an influx of Ca<sup>2+</sup> and was independent of CICR through RyR-3, the activation of which requires a relatively high Ca<sup>2+</sup> concentration (12).

**Ca**<sup>2+</sup> Response in Myocytes Transfected with Mutated RyR-1 cDNA—In double-mutant myocytes transfected with RyR-1 cDNA, a [Ca<sup>2+</sup>] i increase was observed in response to electrical stimulation in the absence of extracellular Ca<sup>2+</sup> (see Fig. 3A). When RyR-2 cDNA was transfected in place of RyR-1, the myocytes failed to respond to electrical stimulation, although they showed spontaneous Ca<sup>2+</sup> oscillations (not shown). These results are in accordance with previous results obtained using RyR-1-deficient myocytes (13, 22).

To determine whether the D2 region is responsible for the functional difference among the RyR subtypes, we constructed four different expression plasmids (pYT-2, -3, -4, and -5) carrying mutated RyR-1 cDNAs. The entire D2 region (amino acid residues 1303–1406) was deleted in pYT-2, whereas either the N-terminal half (1303–1367) or the C-terminal half (1357–1406) of D2 was deleted in pYT-3 and pYT-4, respectively (Fig. 2). In pYT-5 the entire D2 region was replaced by the corresponding sequence of RyR-2 (1316–1400).

In all double-mutant myocytes transfected with any of the mutated RyR-1 cDNAs, the [Ca<sup>2+</sup>] i response to caffeine was recovered (Fig. 3, B–E). Therefore, the mutations in the D2 region had no obvious effect on the Ca<sup>2+</sup> release function and
Fig. 2. Comparison of amino acid sequences of the D2 region of RyR subtypes. The numbers at both ends of the one-letter-coded sequences indicate the amino acid residue numbers (14, 18). Amino acid residues identical to the corresponding residues in RyR-1 are indicated by reversed letters. Gaps (dashes) have been inserted to achieve maximum identity. Overlines indicate the sites deleted in expression plasmids pYT-3 and pYT-4. Both sites were contiguously deleted in pYT-2 or replaced by the corresponding sequence of RyR-2 in pYT-5.

\[ \text{RyR-1} \]
\[ \Delta 1303-1367 \]
\[ 15 \text{~V, 1 ms} \]
\[ 2 \text{~mm Ca}^2+ \]
\[ 25 \text{~mM caffeine} \]

\[ \text{RyR-2} \]
\[ \Delta 1357-1406 \]
\[ 15 \text{~V, 1 ms} \]
\[ 0.5 \text{~mm EGTA} \]
\[ 25 \text{~mM caffeine} \]

\[ \text{RyR-3} \]
\[ \Delta 1357-1406 \]
\[ 15 \text{~V, 1 ms} \]
\[ 2 \text{~mm Ca}^2+ \]
\[ 25 \text{~mM caffeine} \]

\[ \text{RyR-2} \] D2
\[ 15 \text{~V, 1 ms} \]
\[ 0.5 \text{~mm EGTA} \]
\[ 25 \text{~mM caffeine} \]

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