Decreased Expression of Ryanodine Receptors Alters Calcium-induced Calcium Release Mechanism in mdx Duodenal Myocytes*

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It is generally believed that alterations of calcium homeostasis play a key role in skeletal muscle atrophy and degeneration observed in Duchenne’s muscular dystrophy and mdx mice. Mechanical activity is also impaired in gastrointestinal muscles, but the cellular and molecular mechanisms of this pathological state have not yet been investigated. We showed, in mdx duodenal myocytes, that both caffeine- and depolarization-induced calcium responses were inhibited, whereas acetylcholine- and thapsigargin-induced calcium responses were not significantly affected compared with control mice. Calcium-induced calcium release efficiency was impaired in mdx duodenal myocytes depending only on inhibition of ryanodine receptor expression. Duodenal myocytes expressed both type 2 and type 3 ryanodine receptors and were unable to produce calcium sparks. In control and mdx duodenal myocytes, both caffeine- and depolarization-induced calcium responses were dose-dependently and specifically inhibited with the anti-type 2 ryanodine receptor antibody. A strong inhibition of type 2 ryanodine receptor in mdx duodenal myocytes was observed on the mRNA as well as on the protein level. Taken together, our results suggest that inhibition of type 2 ryanodine receptor expression in mdx duodenal myocytes may account for the decreased calcium release from the sarcoplasmic reticulum and reduced mechanical activity.

Dystrophin is a cytoskeletal structural protein present in skeletal, cardiac, and smooth muscles (1). Although it is well established that the lack of dystrophin expression is the primary genetic defect in Duchenne’s muscular dystrophy, functionality of smooth muscles in patients with Duchenne’s muscular dystrophy and in mdx mice has received little attention. However, different degrees of disorders have been observed in mdx smooth muscles of the digestive track (impaired nitricergic relaxation and increase of spontaneous tone, Refs. 2 and 3), and different clinical manifestations, including gastric dilatation and intestinal pseudo-obstructions, have been reported in patients with Duchenne’s muscular dystrophy (4, 5). The role of dystrophin in smooth muscle contraction is still largely unknown.

In skeletal and cardiac muscles, it has been suggested that an elevation in \([\text{Ca}^{2+}]\), under resting conditions may activate \([\text{Ca}^{2+}]\)-dependent proteases inducing muscle damage (1). In fact, some groups have found a difference in \([\text{Ca}^{2+}]\), between normal and dystrophic skeletal muscles from patients and mdx mice (6, 7). Other groups have not been able to confirm these data (8), although an elevated subsarcolemmal \([\text{Ca}^{2+}]\) concentration has been reported by studying activation of \([\text{Ca}^{2+}]\)-activated \(K^+\) channels (9). An increased \([\text{Ca}^{2+}]\) influx through cationic channels has been detected in mdx skeletal fibers, suggesting that a dysregulation of channel activity may be involved in this neuromuscular disorder (10). Controversial data also have been reported for the peak \([\text{Ca}^{2+}]\) responses upon stimulation. Some groups have found larger \([\text{Ca}^{2+}]\) rises in mdx mice (7), others have found them to be similar to controls (11), and some have even reported reductions (12).

A key aspect of the \([\text{Ca}^{2+}]\) signaling pathway is represented by its spatial and temporal complexity. Localized changes in \([\text{Ca}^{2+}]\) are pivotal events in triggering important cellular responses such as contraction, secretion, gene expression, and metabolic activation. In smooth muscle cells, \([\text{Ca}^{2+}]\) release channels of the sarcoplasmic reticulum (SR) modulate the \([\text{Ca}^{2+}]\), in response to activation of voltage-gated \([\text{Ca}^{2+}]\) channels (13) and membrane receptors (14, 15).

In this study, we tested the hypothesis that, in visceral smooth muscle lacking dystrophin, the \([\text{Ca}^{2+}]\) responses evoked by the \([\text{Ca}^{2+}]\)-induced \([\text{Ca}^{2+}]\) release (CICR) mechanism could be affected by the mutation. We addressed this issue by using patch clamp technique coupled to confocal microscopy with F-4 to analyze \([\text{Ca}^{2+}]\) signals, binding experiments, and Western blotting to evaluate the expression of ryanodine receptors (RYRs) in duodenal myocytes from wild-type and mdx mice. We show for the first time that the RYR2 expression is impaired in mdx duodenal myocytes and that this alteration may account for the reduced \([\text{Ca}^{2+}]\) responses evoked by caffeine and activation of voltage-gated \([\text{Ca}^{2+}]\) channels.

EXPERIMENTAL PROCEDURES

Cell Preparation—The investigation conformed with the European Community and French guiding principles in the care and use of animals. Authorization to perform animal experiments (A-33-063-003) was obtained from the Préfecture de la Gironde (France).

Wild-type control (C57BL/10) and mdx (C57BL/10 mdx) mice aged 5–8 months were killed by cervical dislocation. Isolated myocytes were obtained from the longitudinal layer of the duodenum by enzymatic dispersion as described previously (16). Cells were seeded on glass slides in M199 culture medium containing 10% fetal calf serum, 2 mM glutamine, 1 mM pyruvate, 20 units/ml penicillin, and 20 \(\mu\)g/ml strep-

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1The abbreviations used are: SR, sarcoplasmic reticulum; ACh, acetylcholine; CICR, calcium-induced calcium release; RYR, ryanodine receptor; RT, reverse transcription; NSF, nonspecific fluorescence.
tonycin. Cells were kept in an incubator gassed with 95% air and 5% CO₂ at 37 °C and used within 8 h.

Reverse Transcription-Polymerase Chain Reaction—Total RNA was extracted from freshly isolated mouse duodenal smooth muscle cells using an RNeasy minikit (Qiagen, Hilden, Germany) following the instructions of the supplier. The reverse transcription (RT) reaction was performed using a Sensiscrypt RT kit (Qiagen). Total RNA was incubated with oligo(dT) primers (Promega, Lyon, France) at 65 °C for 5 min. After a cooling time of 15 min at 25 °C, RT mixture was added, and the total mixture was incubated for 60 min at 37 °C. The resulting cDNA was stored at −20 °C. The PCR was performed with 1 μg of cDNA, 1.25 units of HotStart Taq DNA polymerase (Qiagen), a 1 μM concentration of each primer, and a 200 μM concentration of each dNTP in a final volume of 50 μl. The reaction conditions were as follows: 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 58 °C for 45 s, and 72 °C for 1 min. The PCR products were separated by electrophoresis (2% agarose gel) and visualized by ethidium bromide staining. The minimum detection of RYR amplification products was obtained with 15 ng of cDNA. Gels were photographed with EDAS 120 and analyzed with KSIDS 2.0 software (Kodak Digital Science, Paris, France). The relative amount of the amplification products was determined and normalized to that of the glyceraldehyde-3-phosphate dehydrogenase fragment. The identity of the PCR products was verified by DNA sequencing.

Sense (s) and antisense (as) primer pairs specific for RYR1, RYR2, and RYR3 were designed on the known cloned receptor sequences and RYR3 were designed on the known cloned receptor sequences deposited in the GenBank™ sequence data base (accession numbers X59392, X59393, and X59394, respectively) with Lasergene software (DNASTAR, Madison, WI). The nucleotide sequence and the length of the expected PCR products (in parentheses), respectively, for each primer pair were as follows: RYR1(s), GAAGGTTCTGGACAAAGAGGTTCTCCAAAAGC (435 bp); RYR2(s), GAATCTGTTAGTATGCGCAGGTAATGCGCAAGGTTCTCCAAAAGC (635 bp); RYR3(s), AGAAGAGGCCAAAAGCAAGGTTCTCCAAAAGC (269 bp) (17).

Western Blot—The longitudinal layer of the duodenum from wild-type and mdx mice was homogenized in an appropriate volume of 10% SDS. After centrifugation (10 min, 2700 rpm), supernatants were collected, and the protein content was measured according to Bradford (18). Equal amounts of protein (50 μg) from wild-type and mdx tissues were heated at 95 °C for 3 min in Laemmli buffer, separated by 6% SDS-polyacrylamide gel electrophoresis, and electrotransferred to polyvinylidene difluoride membranes (70 μm, 100 V, 4 °C). Non-specific binding was blocked by incubating membrane in phosphate buffer/Tween 20 (0.1%) containing 5% nonfat dry milk for 1 h, and blots were incubated (overnight, 4 °C) with anti-RYR1 (1:1000), anti-RYR2 (1:500), or an antibody directed against a specific anti-RYR subtype antibody (1:500) and then cells were washed (2 × 350 ml) and incubated with the appropriate secondary antibody conjugated to fluorescein isothiocyanate for 45 min at room temperature. After washing in phosphate-buffered saline, cells were mounted in Vectashield (Abb-Cys, Paris, France). Images of the stained cells were obtained with a confocal microscope (Bio-Rad MRC1024), and fluorescence was estimated using gray level analysis using Lasersharp software (Bio-Rad) and expressed by volume unit. Cells were compared by keeping acquisition parameters (gray scale, exposure time, iris aperture, gain, laser power, etc.) constant. Non-specific fluorescence (NSF) was determined when specific anti-RYR subtype antibody was preincubated with its antigen peptide for 1 h before application of the immunostaining protocol. When the cell fluorescence obtained with the anti-RYR subtype antibody was higher than NSF, the cell was considered to be immunopositive, and specific fluorescence (F − NSF) was estimated.

[R]Ryanodine Binding Assay—Microsomal membranes from the longitudinal layer of mouse duodenum were prepared by homogenization with a Kontes Potter-Elvehjem pestle in a solution containing 20 mM Tris-HCl, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride, pH 7. The homogenate was centrifuged at 1200 rpm for 10 min at 4 °C. Microsomal membranes were obtained as a pellet by centrifugation of the supernatant at 40,000 rpm for 90 min at 4 °C. Microsomal membranes were then resuspended in 21 mM KCl containing 10 mM EGTA (R_{max}) or after application of a 200-mV hyperpolarizing step causing membrane breakdown (R_{min}). R_{max} and R_{min} values from control (n = 94) and mdx myocytes (n = 95) were 0.36 ± 0.05, 2.4 ± 0.03, 0.38 ± 0.05, and 4.4 ± 0.09, respectively. An intracellular value for the quantity K = K_{p Hi} was determined according to Gryniewicz et al. (21) in control and mdx myocytes (n = 94) defined as R_{max} (at R_{min}/F_{max0} (at R_{max}). K values from control (n = 94) and mdx myocytes (n = 95) were 979 ± 116 and 1357 ± 376 ns, respectively. These parameters were used to calculate the [Ca^{2+}]_{i} values according to Gryniewicz’s formula (22). All measurements were made at 25 °C ± 1 °C.

For saturation experiments, Fluo-4 (50 μM) was diazylized into the cell through the patch clamp pipette. Images were acquired using the line scan mode of a confocal Bio-Rad MRC1024 microscope connected to a Nikon Diaphot microscope. Excitation light was delivered by a 25-milliwatt argon laser (Ion Laser Technology, Salt Lake City, UT) through a Nikon Plan Apo × 60, 1.4 numerical aperture, objective lens. Fluo-4 was excited at 488 nm, and emitted fluorescence was filtered and measured at 522 ± 55 nm. At the setting used to detect Fluo-4 fluorescence, the resolution of the microscope was near 0.4 × 0.4 × 1.5 μm (x, y, and z axis). Scanned lines were plotted vertically, and each line was added to the right of the preceding line to form the line scan image. Fluorescence signals are expressed as pixel per pixel fluorescence ratios (F/F_{0}) where F is the fluorescence during a response and F_{0} is the rest level fluorescence of the same pixel. Image processing and analysis were performed by using Lasersharp 2000 (Bio-Rad) and IDL softwares (Research Systems, Inc., Boulder, CO).

Voltage clamp was made with a standard patch clamp technique using a List EPC-7 patch clamp amplifier (Darmstadt-Eberstadt, Germany). Holding potential (V_{P}) is the fluorescence during a response and P_{E} is the rest level fluorescence of the same pixel. Image processing and analysis were performed by using Lasersharp 2000 (Bio-Rad) and IDL softwares (Research Systems, Inc., Boulder, CO).

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Ca\(^{2+}\) Responses in Duodenal Myocytes from mdx Mice—In control duodenal myocytes from C57BL/10 mice, dystrophin was present and located at the periphery of the cell sections, whereas dystrophin was absent in mdx mice (not shown). In freshly isolated single myocytes from control and mdx mice, the resting [Ca\(^{2+}\)], levels were estimated to be 61 \pm 5 nM (n = 94) and 59 \pm 5 nM (n = 95), respectively, and were not significantly different (p > 0.05). Applications of caffeine (10 mM) or acetylcholine (ACH, 10 \mu M) activated transient increases in [Ca\(^{2+}\)], that have been shown to depend essentially on Ca\(^{2+}\) release from the SR (16). With time intervals of 3 min between successive applications of the stimulating substances, similar Ca\(^{2+}\) responses were obtained in the same cell, indicating complete refilling of the internal Ca\(^{2+}\) store within 3 min (16). As shown in Fig. 1, the caffeine-induced Ca\(^{2+}\) responses were decreased by about 50% in mdx duodenal myocytes, whereas the ACh-induced Ca\(^{2+}\) responses were not significantly reduced when compared with control mouse cells. As caffeine is known as a pharmacological activator of RYRs, we tested the effects of membrane depolarizations on Ca\(^{2+}\)-induced Ca\(^{2+}\) release. In control mouse cells, maximal Ca\(^{2+}\) inward currents in response to depolarizing steps from −70 to 0 mV triggered maximal transient Ca\(^{2+}\) responses (Fig. 2A). In mdx mouse cells, the Ca\(^{2+}\) responses were reduced by about 45%, whereas the Ca\(^{2+}\) currents were similar (Fig. 2, A and B). Quantitative results indicated that Ca\(^{2+}\) current densities evoked by a depolarizing step from −70 to 0 mV were similar in control (13.4 \pm 1.4 pA/pC) and mdx mouse cells (14.1 \pm 1.1 pA/pC), n = 14). This current is due to activation of two different types of calcium channels: a typical L-type calcium channel and a second type resistant to dihydropyridines but inhibited by mapacaline (27). It is noteworthy that, in the presence of a mixture of 1 \mu M oxodipine and 5 \mu M mapacaline for 5 min to block sarcoplasmatic Ca\(^{2+}\) channels, both inward current and increase in [Ca\(^{2+}\)], were suppressed during test depolarizations in control and mdx mice (not shown). As illustrated by the current-voltage relationships in Fig. 2B, the threshold potential and the corresponding to peak current were not different in control and mdx mice. The \Delta[Ca\(^{2+}\)]-voltage relationship revealed that, in mdx mice, the peak Ca\(^{2+}\) responses were significantly reduced in the voltage range from −10 to +20 mV (Fig. 2B). To establish whether mdx mutation may reduce the loading of the intracellular Ca\(^{2+}\) store, we studied the effect of thapsigargin (a SR Ca\(^{2+}\) ATPase inhibitor) to deplete the SR. In myocytes from control mouse cells, application of 1 \mu M thapsigargin (in Ca\(^{2+}\)-free 0.5 mM EGTA-containing solution for 30 s) evoked a sustained increase in [Ca\(^{2+}\)], of 112 \pm 14 nM (n = 11). In mdx mouse cells, the thapsigargin-induced Ca\(^{2+}\) response was not significantly affected (110 \pm 21 nM, n = 13). Taken together, these results indicate that, in mdx mice, the decrease of Ca\(^{2+}\) responses evoked by caffeine and depolarizing steps did not appear to be due to an inhibition of voltage-dependent Ca\(^{2+}\) channels or Ca\(^{2+}\) loading of the SR.

Confocal Ca\(^{2+}\) Signals Evoked by Activation of Voltage-gated Ca\(^{2+}\) Channels and Flash Photolysis of Caged Ca\(^{2+}\)—Spontaneous Ca\(^{2+}\) sparks were not detected in control and mdx duodenal myocytes (n = 155). Various experimental conditions, such as applications of low concentrations of caffeine or Bay K 8644 (an L-type Ca\(^{2+}\) channel agonist) or low membrane depolarizations, have been reported to trigger and increase the frequency of Ca\(^{2+}\) sparks in vascular myocytes (17). Applications of 5 mM Bay K 8644 (n = 79), low depolarizations (from −70 to −50 mV or from −50 to −20 mV, n = 41), or 1 mM caffeine (n = 35) were ineffective in inducing generation of Ca\(^{2+}\) sparks in control and mdx mice. In contrast, depolarizing steps applied from −70 to 0 mV elicited propagated Ca\(^{2+}\) waves. As shown in Fig. 3, A and B, Ca\(^{2+}\) responses evoked by depolarizing steps from −70 to −30 mV were not statistically different in control and mdx mice, whereas Ca\(^{2+}\) responses evoked by higher depolarizing steps (from −70 to 0 mV) were reduced by about 40% in mdx mouse cells compared with control cells (Fig. 3, A and B). These results show that inhibition of Ca\(^{2+}\) responses in mdx myocytes can also be detected in line scan images.

RYRs can be directly activated by an increase in [Ca\(^{2+}\)] in the vicinity of the receptors as demonstrated previously in vascular myocytes (13). Flash photolysis of DM-nitrophen (caged Ca\(^{2+}\)) instantaneously elevated (within 2 ms) the Ca\(^{2+}\)
concentration and evoked Ca\textsuperscript{2+} transients in the entire line scan image (Fig. 4A). Plotting the peak of the Ca\textsuperscript{2+} transients as a function of flash intensity revealed that the Ca\textsuperscript{2+}-induced increase in [Ca\textsuperscript{2+}], in mdx mice was significantly reduced compared with control mice, particularly for high UV flash intensities (Fig. 4B). The Ca\textsuperscript{2+} sensitivity of RYRs can be estimated by plotting the ratio between the peak Ca\textsuperscript{2+} transients and the maximal Ca\textsuperscript{2+} transient at different UV flash intensities for control and mdx mice. The points appeared to be superimposed suggesting no changes in the Ca\textsuperscript{2+} sensitivity of RYRs in mdx mice (Fig. 4C).

Applications of 10 mM caffeine evoked propagating Ca\textsuperscript{2+} waves in duodenal myocytes from control and mdx mice. The peak amplitude of these responses was reduced by about 40% in mdx compared with control mice, particularly for high UV flash intensities (Fig. 4B). Myocytes were loaded with Indo-1 and held at −70 mV. Data are means ± S.E. for three to seven cells in control (●) and mdx duodenal myocytes (○). Myocytes were loaded with Indo-1 and held at −70 mV. *, p < 0.05.

Fig. 2. Ca\textsuperscript{2+} current and increase in [Ca\textsuperscript{2+}], as a function of membrane potential in control and mdx mice. A, typical recordings obtained from control and mdx duodenal myocytes in response to depolarizing steps from −70 to 0 mV. B, Ca\textsuperscript{2+} current and Δ[Ca\textsuperscript{2+}], against membrane potential. Holding potential, −70 mV. Data are means ± S.E. for three to seven cells in control (●) and mdx duodenal myocytes (○). Myocytes were loaded with Indo-1 and held at −70 mV. *, p < 0.05.

To confirm that the decrease in RYR2 expression could be responsible for the reduced Ca\textsuperscript{2+} responses in mdx mice, we specific [\textsuperscript{3}H]ryanodine binding (Fig. 5B) revealed that the maximal binding capacity decreased by about 40% (control: 633 ± 47 fmol/mg of protein, n = 4; mdx: 382 ± 14 fmol/mg of protein, n = 4), whereas the dissociation constants were similar in control (K\textsubscript{d} = 12.3 ± 0.4 nM, n = 4) and mdx mice (K\textsubscript{d} = 9.5 ± 0.5 nM, n = 4). These results support the idea that the density of RYRs was decreased in mdx duodenal myocytes.

Fig. 3. Ca\textsuperscript{2+} responses evoked by various depolarizing steps in control and mdx duodenal myocytes. A, effects of depolarizing steps (from −70 to −30 or 0 mV) on line scan fluorescence images from control and mdx mice. Note the absence of any Ca\textsuperscript{2+} sparks. B and C, peak amplitude (Δ(F/F\textsubscript{0})) in control (open bars) and mdx duodenal myocytes (hatched bars) with the number of cells tested indicated in parentheses. Myocytes were loaded with Fluo-4 and held at −70 mV. Depolarizing steps to −30 mV (B) or 0 mV (C) were used. *, p < 0.05.
tested the effects of both anti-RYR2 and anti-RYR3 antibodies on duodenal \( \text{Ca}^{2+} \) responses. Concentration-dependent inhibitory effects and specificity of these antibodies have been reported previously in other smooth muscle cells (25, 28). In both control and mdx mice, intracellular applications of 10 \( \mu \text{g/ml} \) anti-RYR3 antibody for 7 min had no significant effect on the caffeine-induced \( \text{Ca}^{2+} \) responses (Fig. 8). In contrast, the anti-RYR2 antibody inhibited in a concentration-dependent manner the \( \text{Ca}^{2+} \) responses in control mice (Fig. 8A) as well as in mdx mice (Fig. 8B). The specificity of the anti-RYR2 antibody was confirmed by the absence of effect of the antibody when it was preincubated with its peptide epitope before intracellular application (Fig. 8). We also found that the \( \text{Ca}^{2+} \) responses evoked by depolarizing steps (from \(-70\) to \(0\) mV) in both control and mdx duodenal myocytes were selectively inhibited by the anti-RYR2 antibody \((n = 7)\), whereas they were unaffected by the anti-RYR3 antibody \((n = 5)\).

**DISCUSSION**

In Duchenne’s muscular dystrophy patients and mdx mice, it is generally accepted that the missing link between the absence of dystrophin and muscle hypotonia is due to an alteration in \( \text{Ca}^{2+} \) homeostasis, but no firm conclusions have yet been reached. In the gastrointestinal track, impaired nitricergic relaxation and increase of spontaneous tone have been reported in mdx mice (2, 3). We have readdressed this issue by meas-
uring [Ca\(^{2+}\)] in localized areas with confocal microscopy, voltage-gated Ca\(^{2+}\) currents and global Ca\(^{2+}\) release from the SR, and ryanodine receptor expression in duodenal myocytes from control and mdx mice.

Under resting conditions, no significant difference in bulk cytosolic [Ca\(^{2+}\)], between mdx and control mice was observed in duodenal myocytes as well as in line scan images using two different Ca\(^{2+}\) dyes in agreement with previous data obtained in vas deferens (29). In addition, no variation in SR Ca\(^{2+}\) loading was detected as illustrated by the absence of significant reduction in ACh-induced Ca\(^{2+}\) release at a concentration of ACh (10 \(\mu\)M) that had been shown to completely deplete the SR (16). Capacitive Ca\(^{2+}\) entry was not affected in mdx duodenal myocytes as revealed by similar thapsigargin-induced Ca\(^{2+}\) responses obtained in control and mdx mice. In contrast, both caffeine- and depolarization-induced Ca\(^{2+}\) responses were significantly inhibited in mdx compared with control duodenal myocytes. The reduction of depolarization-induced Ca\(^{2+}\) responses was not dependent on a reduced voltage-gated Ca\(^{2+}\) current as the current densities in mdx and control mice were similar. These results were therefore consistent with the possibility that Ca\(^{2+}\) influx was less able to activate the CICR mechanism and/or with a reduction in RYR expression in mdx duodenal myocytes. Ca\(^{2+}\) responses evoked by flash photolysis of caged Ca\(^{2+}\) were reduced in mdx duodenal myocytes, but the Ca\(^{2+}\) sensitivity of these responses, illustrated by the normalized curves of Ca\(^{2+}\) release versus flash photolysis intensity, was not different in mdx compared with control mice suggesting that the gain of function of the CICR was not altered. In contrast, RYR expression was reduced in mdx mice as the maximal binding capacity of \(^{3}H\)ryanodine to duodenal membranes was strongly decreased, suggesting that the reduction of RYRs might account for the diminished Ca\(^{2+}\) responses to caffeine and voltage-gated Ca\(^{2+}\) currents. However, three RYR subtypes are generally expressed in smooth muscles, and their roles as functional Ca\(^{2+}\) release channels have been questioned (17, 28).

Duodenal myocytes expressed both RYR2 and RYR3 isoforms but not RYR1. This is in contrast with vascular myocytes that express all three RYR subtypes (17) and non-pregnant myometrial cells that express only RYR3 (28). At physiological extracellular Ca\(^{2+}\) concentration, RYR3 is insensitive to caffeine in non-pregnant myometrial cells. In vascular myocytes, both RYR1 and RYR2 are required for triggering Ca\(^{2+}\) sparks and Ca\(^{2+}\) waves induced by activation of L-type Ca\(^{2+}\) current (17). Our results show that both RYR2 and RYR3 are unable to induce Ca\(^{2+}\) sparks in control duodenal myocytes, supporting our model that co-expression of RYR1 and RYR2 is needed to trigger elementary Ca\(^{2+}\) signals in smooth muscle. In duodenal myocytes, Ca\(^{2+}\) responses are dependent on the expression of RYR2 as the anti-RYR2-specific antibody inhibited in a concentration-dependent manner the caffeine- and the depolarization-induced Ca\(^{2+}\) waves. In contrast, the anti-RYR3-specific antibody was ineffective, indicating that, at physiological extracellular Ca\(^{2+}\) concentration, RYR3 did not participate in the CICR mechanism in duodenal myocytes in agreement with previous data obtained in vascular myocytes (17). In both control and mdx mice, caffeine- and depolarization-induced Ca\(^{2+}\) waves revealed the same sensitivity to the anti-RYR2-specific antibody and the same absence of effects of the anti-RYR3-specific antibody, suggesting that inhibition of RYR2 expression was responsible for the reduced Ca\(^{2+}\) responses observed in mdx duodenal myocytes. These results
were supported by the specific inhibition of RYR2 expression in duodenum of mdx mice as revealed by both RT-PCR and Western blot experiments. This is the first data showing that inhibition of RYR2 expression may be involved in dystrophic smooth muscle. However, a mutation of the RYR1 gene has been reported in human congenital myopathies, resulting in reduced level of SR Ca\(^{2+}\) release (30).

The absence of dystrophin generates modifications of ion channels that may lead to alterations of Ca\(^{2+}\) flux. Changes of Ca\(^{2+}\) channel activity or appearance of novel forms of cationic channels have been reported in dystrophic muscles (10, 31). It has been speculated that the lack of dystrophin in skeletal muscle may induce localized structural disorders leading to disrupted excitation-contraction coupling in relation to chronic muscle may induce localized structural disorders leading to disrupted excitation-contraction coupling in relation to chronic muscle. Further experiments are necessary that are beyond the scope of this work to establish the sequence of events from the lack of dystrophin to alterations of protein expression, particularly of RYR isoforms, and to correlate inhibition of RYR expression with disorders of CICR and contraction in other muscles.

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