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Contractile impairment and structural alterations of skeletal muscles from knockout mice lacking type 1 and type 3 ryanodine receptors

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Abstract Skeletal muscle contraction is triggered by the release of Ca^{2+} from the sarcoplasmic reticulum through the type 1 ryanodine receptor (RyR1). Recently it has been shown that also the type 3 isoform of ryanodine receptor (RyR3), which is expressed in some mammalian skeletal muscles, may participate in the regulation of skeletal muscle contraction. Here we report the generation and the characterization of double mutant mice carrying a targeted disruption of both the RyR1 and the RyR3 genes ($RyR1^{-/-}$;$RyR3^{-/-}$). Skeletal muscles from mice homozygous for both mutations are unable to contract in response to caffeine and to ryanodine. In addition, they show a very poor capability to develop tension when directly activated with micromolar $[Ca^{2+}]_i$ after membrane permeabilization which indicates either poor development or degeneration of the myofibrils. This was confirmed by biochemical analysis of contractile proteins. Electron microscopy confirms small size of myofibrils and shows complete absence of feet (RyRs) in the junctional SR.

Key words: Knockout mouse; Skeletal muscle contraction; Ryanodine receptor, type 1 and type 3; Prenatal muscular development

1. Introduction

Excitation-contraction (E-C) coupling mediates muscle contraction in response to electrical stimulation. The skeletal muscle ryanodine receptor (RyR1) in the sarcoplasmic reticulum (SR) and the slow voltage-gated calcium channel in the surface membrane (DHPR) are two important proteins associated with this process. E-C coupling is lost in skeletal muscles of mice that carry targeted non-functional alleles of either the skeletal ryanodine receptor (RyR1) or the α1 subunit of the dihydropyridine receptor [1–4]. This demonstrates, therefore, that both proteins are essential components of the mechanism which links plasma membrane depolarization with release of Ca^{2+} from the SR. An additional isoform of the ryanodine receptor, the RyR3 isoform, has been detected, in low amounts, in some mammalian skeletal muscles [5–7]. Studies on RyR1 knockout mice have shown that muscles from these mice were still somewhat responsive to caffeine stimulation [1,8,9]. This raised the possibility that RyR3 may participate in the regulation of skeletal muscle contraction. The mechanism for this interaction would be through a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism of signal amplification by RyR3 in conjunction with RyR1. A specific role for RyR3 is also supported by studies on skeletal muscles from RyR3 knockout mice [10]. Although the skeletal muscle from these mice was shown to retain normal E-C coupling, it showed a reduced CICR response at high Ca^{2+} concentrations compared to muscles of wild-type mice. RyR3 appears to occur in murine skeletal muscles only during the postnatal phase of muscle development, and it is only detectable in diaphragm and soleus muscles of the adult. This is at variance with the more homogeneous expression of RyR1, with relation to age and muscle type. Skeletal muscles of RyR3 knockout mice were found to have a reduced response to electrical stimulation and to caffeine during the first weeks after birth in accordance with the observed neonatal expression of the RyR3 [11].

In a recent study [12] Ca^{2+} release was studied with the fluorescent indicator fura-2 in permeabilized fiber segments dissected from hindlimb skeletal muscles of mice lacking both RyR1 and RyR3. In these muscles both the response to electrical stimulation and the response to caffeine were lost. Furthermore, severe muscular degeneration was observed. In view of the fact that the absence of both RyR1 and RyR3 seems to impair CICR and produce disarray of the contractile apparatus of skeletal muscles, we thought it was of interest to study the contractile performance of such muscles. In this study we determined the tension development of muscles and skinned muscle fiber bundles from mice lacking either RyR1 or RyR3 or both. Intact skeletal muscles from mice homozygous for both mutations failed to show a response to caffeine or ryanodine, indicating the absence of all ryanodine/caffeine sensitive pathways of Ca^{2+} release. In addition, these muscles, after chemical skinning and direct activation with micromolar Ca^{2+} concentration, developed less tension than muscles lacking only one of the two RyR isoforms. This loss of contractile response was roughly proportional to the reduction in myofibrillar protein content. Our results suggest that the RyR3 channel might have a physiological role in prenatal muscular development, in addition to its recently demonstrated role in the regulation of muscle contraction during the first postnatal weeks [11].

2. Materials and methods

The method of generation of $RyR1^{-/-}$ and $RyR3^{-/-}$ knockout

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mice is described elsewhere [2,9,11]. Double mutant mice were obtained by mating mice heterozygous at the RyR1 locus and homozygous for the disrupted allele at the RyR3 locus (i.e. $RyR1^{+}$; $RyR3^{-/-}$). All the double knockout neonates were confirmed by the polymerase chain reaction and Southern blot for the analysis of RyR1 and RyR3 loci respectively.

The contractile properties of skeletal muscle were studied in diaphragm preparations obtained from mutant and wild-type mice immediately after birth. Diaphragm strips which included the central tendon and the right and left rib insertions were dissected while immersed in oxygenated Krebs solution under a stereomicroscope (×10-×40, Wild). The width of the strips, measured in the stereomicroscope at 40×, ranged between 1.87 and 3.25 mm whereas the thickness was between 0.075 and 0.15 mm and the rib to rib distance ranged between 4.3 and 6.4 mm. The preparations were transferred to the myograph where they were mounted between the hook of a force transducer (AE 801Sensonor Horten, Norway) and a movable shaft by means of small holes opened between the ribs and strengthened with silk ligatures. The perfusion bath (volume 2 ml) was filled with bicarbonate Krebs solution, bubbled with O_2-CO_2 mixture and kept at constant temperature (22°C). The perfusing solution could be quickly renewed (5–10 s) by flushing approximately 20 ml of new solution through the chamber. On both sides of the perfusion bath at a distance of about 2 mm from the preparations, platinum plate electrodes connected with a stimulator (Grass S48, Quinci, MA, USA) allowed field electrical stimulation. The output of the tension transducer was stored digitally after A/D conversion and recalled for analysis (CEA 1401 A/D converter and CEA Spike2 software-CEA Cambridge, UK).

The preparations were stretched just above slack length and their response to electrical stimulation was tested. If a response could be elicited, the preparation was stimulated for about 30 min with supramaximal, low frequency (0.1 Hz) stimuli. Then the response to twitch and short tetanic stimuli (0.5 s duration, 5, 10, 20, 50 Hz frequency) were recorded. If there was no response, the pharmacological tests were immediately begun (see below).

After the electrical responses, the response to pharmacological agents able to release Ca^{2+} (30 mM caffeine or, alternatively, 10 μM ryanodine) was tested. The solution in the chamber was quickly replaced with a new one containing the selected agent and, once the contracture had fully developed, the preparation was washed with Krebs solution without caffeine or ryanodine.

The ability of the myofibrils to develop tension in response to direct Ca^{2+} activation of the myofibrils was also tested in all preparations. To this end the preparation was immersed for 1 h in skinning solution containing EGTA and Triton X-100 at low temperature (10°C) to remove the membranes. The solution in the chamber was then replaced with a relaxing solution at p*Ca* 8. After a few minutes the preparation was activated to develop tension by quickly filling the chamber with activating solution (p*Ca* = 4.5). When tension reached a stable level, the preparation was relaxed by filling the chamber with relaxing solution. The composition of skinning, relaxing and activating solutions has been previously described [13]. All force measurements are reported as mean ± S.E.M. Statistical significance of the differences between the four groups of preparations was assessed by variance analysis followed by Student-Newman-Keuls test.

For Western blot analysis whole diaphragm muscles were homogenized as previously described [6]. Protein concentration was established by Bradford analysis. SDS-PAGE was performed on 7.5% gels for MHC, 10% gels for SERCA1 and calsequestrin, 12.5% or 15% gels for TnT and TnI (acrylamide: bis 30:0.8). Bound antibodies were detected with alkaline phosphatase detection method or with the ECL technique.

Transmission electron microscopy analysis of skeletal muscle samples was performed as previously described [14].

3. Results

The results of the analysis of the contractile response of the four groups of muscles at birth ($RyR1^{+/+}$;$RyR3^{+/+}$, $RyR1^{+/+}$;$RyR3^{-/-}$, $RyR1^{-/-}$;$RyR3^{+/+}$, $RyR1^{-/-}$;$RyR3^{-/-}$) are summarized in Fig. 1. Perfusion of the diaphragm strips with activating solution (p*Ca* = 4.5) after chemical skinning allowed direct and maximal Ca^{2+} activation of the myofibrillar apparatus, bypassing E-C coupling and any other process of Ca^{2+} release from internal stores. In $RyR1^{+/+}$;$RyR3^{-/-}$ mice the amplitude of the response to direct activation was the same as in $RyR1^{+/+}$;$RyR3^{+/+}$ mice. Thus the lack of RyR3 did not have any effect on force development. On the contrary, in the muscles from the $RyR1^{-/-}$;$RyR3^{+/+}$ mice the force developed during direct and maximal activation was 14% of that developed in wild-type muscles and in $RyR1^{-/-}$;$RyR3^{-/-}$ mice it dropped to only 7% of control (Fig. 1A).

Electrical stimulation was able to produce a contractile response only in $RyR1^{+/+}$;$RyR3^{+/+}$ and $RyR1^{+/+}$;$RyR3^{-/-}$ mice (i.e. in the preparations from the mice where the RyR1 isoform was expressed). Force developed during fused tetani was similar in both preparations (Fig. 1B) and somewhat greater than force developed in the same muscles by direct activation after chemical skinning. Insufficient penetration of skinning and of activating solutions might explain the difference with the response to supramaximal electrical stimulation which can reach all fibers. As alternative explanation, internal movement and dys-homogeneity inside fibers might be greater

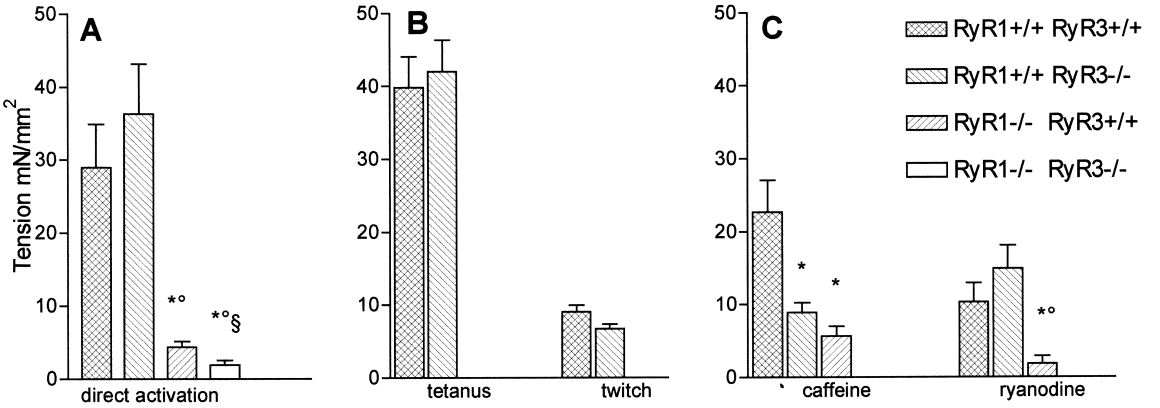

Fig. 1. Contractile responses of diaphragm strips from the four groups of preparations: $RyR1^{+/+}$;$RyR3^{+/+}$ (n = 14), $RyR1^{+/+}$;$RyR3^{-/-}$ (n=13), $RyR1^{-/-}$;$RyR3^{+/+}$ (n = 8), $RyR1^{-/-}$;$RyR3^{-/-}$ (n = 8). A shows the response of all four types of preparations to direct activation with Ca^{2+} (p*Ca* = 4.5) after chemical skinning. B shows the response to electrical stimulation (tetanus 0.5 duration, 50 Hz frequency and twitch 0.1 Hz frequency) of the two types of preparations which contract after electrical stimulation. C shows the response to chemical stimulation of Ca^{2+} release with caffeine (30 mM) and ryanodine (10 μM). Means and standard errors. * indicates significantly different from $RyR1^{+/+}$;$RyR3^{+/+}$, ○ indicates significantly different from $RyR1^{+/+}$;$RyR3^{-/-}$, § indicates significantly different from $RyR1^{-/-}$;$RyR3^{+/+}$.

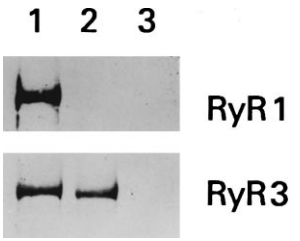

Fig. 2. Western blot analysis of RyR1 and RyR3 isoforms in diaphragm muscle homogenate from different knockout mice.

with the diffusion-limited chemical activation than with the instantaneous electrical stimulation and might reduce tension development. Twitch tension elicited by low frequency stimulation was 30% lower in $RyR1^{+/+};RyR3^{-/-}$ than in $RyR1^{+/+};RyR3^{+/+}$ muscles in agreement with previous observations [11]. The twitch/tetanus ratio in the two groups was 0.23 ± 0.02 ($n = 13$) and 0.16 ± 0.02 ($n = 14$) respectively ($P = 0.033$).

Caffeine (30 mM) and ryanodine (10 μM) induced contractures were observed in all types of preparations, except $RyR1^{-/-};RyR3^{-/-}$ (Fig. 1C). The response to caffeine was fast and large in wild-type preparations, reaching a plateau level of about 80% of tetanic tension in few seconds. This response is much greater than caffeine contracture which can be produced in adult mammalian muscles and is comparable to those observed in amphibian muscles [15]. The absence of RyR3 caused a reduction of the response to caffeine to about 30% of the amplitude in wild-type mice. The absence of RyR1 also caused an even larger reduction in the response to caffeine, and the knockout of both isoforms abolished the response completely. These data demonstrate conclusively that ryanodine receptor expression is necessary for the presence of a caffeine response.

In wild-type mice, 10 μM ryanodine induced a slow contracture which reached a plateau at about 30% of tetanic tension. The response to ryanodine of muscles of mice lacking RyR3 was similar to that of control mice, whereas the ryanodine response of muscles lacking RyR1 was much lower than that of wild-type and of RyR3 knockout mice. The response to ryanodine was totally abolished in muscles of mice carrying double knockout. It is not clear why mice lacking RyR3 show a reduction in their response to caffeine similar to the reduction seen in mice lacking RyR1, while they are significantly different in their response to ryanodine. It is possible that this difference may underlay differences in the contribution of the two isoforms to the mechanisms of Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle cells with RyR3 being more adapted than RyR1 in the CICR process activated by caffeine.

Western blot analysis of diaphragm muscle proteins, using polyclonal antibodies specific for the RyR1 and for the RyR3 proteins [5], indicated the presence of the RyR1 protein in the diaphragm muscle from $RyR1^{+/+};RyR3^{+/+}$ mice, but failed to detect the protein in either the $RyR1^{-/-};RyR3^{+/+}$ (Fig. 2, lane 2) or the double mutant mice (Fig. 2, lane 3). The RyR3 isoform was found to be expressed in both normal and $RyR1^{-/-};RyR3^{+/+}$ mice (Fig. 2, lanes 1 and 2) but not in double knockout mice (Fig. 2, lane 3).

The presence of additional proteins critical for normal muscle function, such as myosin heavy chain (MHC), troponin T (TnT), troponin I (TnI), SERCA1 and calsequestrin was analyzed. The amount of myosin heavy chain (MHC) was investigated by Coomassie staining (not shown) and Western blot, and the presence of troponin T (TnT), troponin I (TnI), SERCA1 and calsequestrin (CSQ) was probed by Western blot analysis. As shown in Fig. 3A, a significant reduction in MHC content was observed in the diaphragm muscle prepared from the $RyR1^{-/-};RyR3^{+/+}$ mice (lane 2) compared to the wild-type preparation (lane 1). An even stronger reduction was detected in the diaphragm from double mutant mice (lane 3). The amount of TnT (Fig. 3B) and TnI (Fig. 3C) was also reduced in the $RyR1^{-/-};RyR3^{+/+}$ and in the double knockout preparations compared to wild-type, while there was no significant difference among preparations in the SERCA1 (Fig. 3D) and CSQ blots (not shown).

Electron microscopy investigation of the double mutant diaphragm muscle showed developmental defects similar to those previously described for both the $RyR1^{-/-};RyR3^{+/+}$ mouse [8] and more recently for the $RyR1^{-/-};RyR3^{-/-}$ mouse [12]. As shown in Fig. 4A, the myofibrils are often branched and poorly developed and the cross striation is misaligned. In cross sections (not shown) the myofibril outlines are not clearly delineated and the myofibrils are less clearly separated from each other than in normal muscle. In the preparation from the $RyR1^{+};RyR3^{-/-}$ mouse, some triads are normal: they contain feet and have a fairly wide junctional gap (Fig. 4B and C). As expected in newborn mouse muscles, other triads are dispedic, resembling those in the $RyR1^{-/-};RyR3^{+/+}$ mouse, in which feet are absent and the junctional gap is smaller. In the double mutant (Fig. 4E and G), triads and dyads are present, although less frequent than in normal muscle, and they all lack feet. Three important features of the SR-T tubule junctions in the double knockouts are: (1) the junctional gap separating the SR and T tubules is of fairly constant width, indicating that some docking protein is holding the two membranes together; (2) the dense content of the terminal cisternae of the SR participating in the junction is closely apposed to the SR membrane and it is condensed in proximity of the membrane similarly to normal muscle (compare Fig. 4B and C with E–G), indicating that the RyRs are not necessary for the anchoring of CSQ to the junctional SR membrane; (3) the junctions are dyspedic (i.e. feet are not visible) and the junctional gap is narrower than in feet-containing junctions of wild-type and all types of $RyR1^{+}$ animals; (4) unlike the $RyR1^{-/-};RyR3^{+/+}$ mouse, there are no rare junctional densities which resemble foot structures. The width of the junctional gap, measured at 192 randomly selected sites in 51 feet-containing junctions from the diaphragm of a RyR1 heterozygous and RyR3 null homozygous

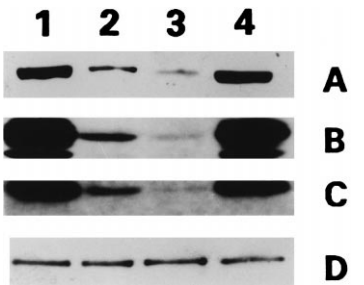

Fig. 3. Western blot analysis of myosin heavy chain, troponin T, troponin I and SERCA1 isoform in diaphragm muscle homogenates from different knockout mice.

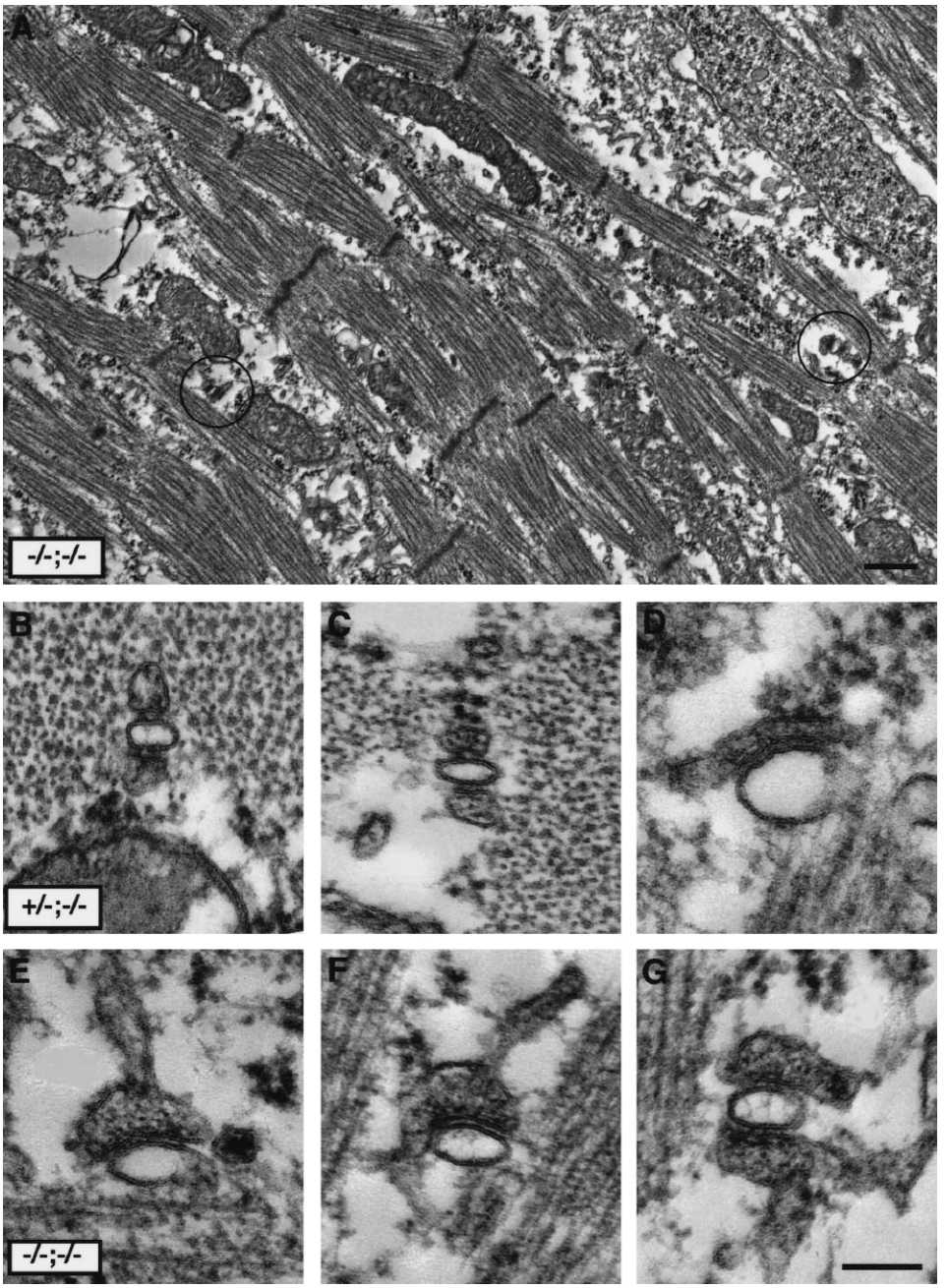

Fig. 4. Electron microscopy investigation of diaphragm muscle. A: Thin longitudinal section from a double mutant at embryonal day 17 (E17), showing myofibrils with frequent branches, lack of cross alignment and a disordered appearance. Two triads are circled. ×20 000, bar = 0.5 micron. B–D: Triads and diads in the diaphragm of a E17 mouse heterozygous at the RyR1 locus and homozygous for the RyR3 knockout ($RyR1^{+}$;$RyR3^{-/-}$). Normal feet-containing triads with fairly wide junctional gap (B–C) are present together with triads and diads with a narrow junctional gap and no visible feet (D). ×140 000, bar = 0.1 micron. E–F: Dyspedic triads and dyads from the diaphragm of a double mutant E17 mouse ($RyR1^{-/-}$;$RyR3^{-/-}$). The junctions have a narrow junctional gap and no feet are present. Calsequestrin in the SR lumen is condensed into periodic densities in proximity of the junctional membrane. ×140 000, bar = 0.1 micron.

mouse is 10.0 ± 1.3 nm (mean ± 1 S.D.), and the width in the dispedic junctions from the double knockout mouse is 5.3 ± 2 nm (from 168 measurements, 39 junctions). The difference is extremely significant ($P < 0.0001$).

4. Discussion

Availability of mice in which both the RyR1 and RyR3 genes have been disabled has allowed a clear analysis of the contribution of these two Ca^{2+} release channels to skeletal muscle development and contractile performance. The main result of this study was that tension development of diaphragm strips directly activated with Ca^{2+} after membrane permeabilization was strongly reduced in $RyR1^{-/-}$;$RyR3^{+/+}$ and even more in $RyR1^{-/-}$;$RyR3^{-/-}$ animals, whereas it was not reduced in the $RyR1^{+/+}$;$RyR3^{-/-}$ animals. This decreased ability to develop force finds a structural explanation in our data as Western blot analysis indicates a reduced content of myofibrils and myofibrillar proteins such as myosin. Interestingly, force measurement and Western blots agree that

the reduction is about two times greater when the lack of RyR1 is accompanied by the lack of RyR3.

In accordance with previous observations [8,10] any impact on myofibrillar development caused by the lack of RyR3 seems to be completely compensated by RyR1. On the other side, the lack of RyR1 has a heavy impact on myofibrillar development in spite of the presence of RyR3. This is not surprising as the RyR1 knockout causes interruption of E-C coupling and of the response to any electrical (and therefore nerve) stimulation ([2,8] and present data). The fact that the functional and structural impairment was even more pronounced when both RyR1 and RyR3 were lacking represents a clear indication, at least in the absence of RyR1, that RyR3 does have some effect on myofibril organization. How this effect is mediated is not clear. However, independent of the mechanisms involved, the developmental defects observed in muscle fibers of mice lacking either RyR1 alone or both RyR1 and RyR3, indicate that both of the two Ca^{2+} release channels provide a pathway of Ca^{2+} release. Furthermore, this also appears to be relevant to myofibril formation and/or survival. Interestingly, the results of this study show that the content of proteins specific to the sarcoplasmic reticulum and the ultrastructural features of the triad itself (with the exception of the width of the intramembrane gap) were not altered in double knockout muscles.

The second important result of this study is the differential response to electrical stimulation and to caffeine and ryanodine among the different mutations. Although tension represents only an indirect indication of Ca^{2+} release, the present study provides useful information on E-C coupling. In the first place this study confirms that in the absence of RyR1, the response to electrical stimulation is lost [2,8]. Furthermore it shows that in the absence of RyR3, the response to twitch stimulation is significantly reduced while tetanic tension is unchanged [11].

The response to caffeine and ryanodine were completely abolished in the absence of both RyR1 and RyR3, indicating that the action of both compounds requires the presence of RyRs to induce Ca^{2+} release. Interestingly, the muscles lacking RyR3 showed a reduced response to caffeine compared to wild-type. This is in agreement with previous observations [11], but the response to ryanodine in these muscles was similar to wild-type muscles. This apparent difference might be explained by assuming that the response to ryanodine is proportional to the number of ryanodine receptors which are blocked in partially open position by 10 μM ryanodine [16] and that the effect of RyR3 knockout on the total number of ryanodine receptors is very small, in agreement with the evidence that RyR3 constitutes not more than 1% of total ryanodine receptors in mammalian diaphragm [17]. On the other hand, the more significant reduction in the caffeine response of RyR3 knockout mice suggests that the RyR3 channels may play a significant role in the CICR process, a role for which they may be more specialized than RyR1 channels [11,18,19]. It is also important to consider that the contractile response of muscles lacking RyR1 to caffeine and ryanodine was small both due to the reduced Ca^{2+} release, caused by the loss of channels, and to the loss of myofibrillar proteins and, therefore, of ability to develop tension.

Electron microscopy of calcium release units in double knockout mice shows that they form even in the absence of both isoforms of RyR demonstrating that neither RyR1 nor RyR3, are involved in the junction formation. Clearly proteins other than RyRs are responsible for the SR-exterior membranes docking. The complete absence of feet in calcium release units of $RyR1^{-/-};RyR3^{-/-}$ mice (see Fig. 4) indirectly suggests that the rare densities previously observed in $RyR1^{-/-};RyR3^{+/+}$ mice [8] likely represent RyR3. In addition, calsequestrin localization in the lumen of SR terminal cisternae is not affected by the total lack of feet structures. The observed lack of feet in some of the junctions in the mouse heterozygous for RyR1, and lacking RyR3 may be simply evidence of a developmental stage in the formation of the junctions.

In conclusion our results show that the lack of RyR1 leads not only to a loss of the E-C coupling process in response to electrical stimulation but also to a loss of the myofibrillar structure and function. The fact that muscle degeneration is enhanced by the simultaneous lack of RyR3 suggests a possible role of RyR3 during muscle development, in addition to its recently demonstrated role in the regulation of contractile response in neonatal muscles [11].

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