

Defective maintenance of intracellular Ca²⁺ homeostasis is linked to increased muscle fatigability in the MG29 null mice

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ABSTRACT

Mitsugumin 29 (MG29) is a transmembrane protein that is normally found in the triad junction of skeletal muscle. Our previous studies have shown that targeted deletion of *mg29* from the skeletal muscle resulted in abnormality of the triad junction structure, and also increased susceptibility to muscle fatigue. To elucidate the basis of these effects, we investigated the properties of Ca²⁺-uptake and -release in toxin-skinned Extensor Digitorum Longus (EDL) muscle fibers from control and *mg29* knockout mice. Compared with the control muscle, submaximal Ca²⁺-uptake into the sarcoplasmic reticulum (SR) was slower and the storage of Ca²⁺ inside the SR was less in the mutant muscle, due to increased leakage process of Ca²⁺ movement across the SR. The leakage pathway is associated with the increased sensitivity of Ca²⁺/caffeine -induced Ca²⁺ release to myoplasmic Ca²⁺. Therefore, the increased fatigability of mutant EDL muscles can result from a combination of a slowing of Ca²⁺ uptake, modification of Ca²⁺-induced Ca²⁺ release (CICR), and a reduction in total SR Ca²⁺ content.

Keywords: MG29, mutant, skeletal muscle, skinned fibers, ECC, Ca²⁺ uptake, CICR.

INTRODUCTION

In skeletal muscle, a functional unit of excitation-contraction coupling (ECC) involves the triad junction formed by the transverse tubule and junctional SR membranes, where the dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR) proteins interact with each other [1]. The DHPR works as the voltage sensor across the sarcolemmal membrane [2] and the RyR functions as the Ca²⁺ release channel in the SR [3, 4]. A number of accessory proteins (e.g. calmodulin, FKBP12, triadin, sorcin, junctin, calsequestrin) have been identified and proposed to be involved with the regulation and modulation of the RyR Ca²⁺ release channel [5].

Mitsugumin 29 (MG29), a transmembrane protein expressed in the triad junction of skeletal muscles [6], has also been proposed to participate in the ECC process of skeletal muscle [7, 8]. The primary amino acid sequence of MG29 exhibits a high degree of homology to synap-

tophysins, a group of membrane proteins with presumed roles in neurotransmitter release [9]. It has been shown [7] that while *mg29* knockout mice survive, reproduce, and show no abnormalities of health under normal housing conditions, extensor digitorum longus (EDL) muscles isolated from the knockout mice produced a slightly lower twitch force but equal peak tetanic force when compared with muscles from wild-type mice. A hallmark feature of skeletal muscles from these mutant mice is the presence of morphological abnormalities of membrane structures around the triad junction, characterized by swollen T-tubule and fragmented SR network [7]. This observation suggested that MG29 is not only important for the proper organization of both the intracellular and cell surface membranes, but also for signal transduction in ECC.

We have previously tested whether the defective membrane structures in the *mg29* knockout mice have an impact on the susceptibility of skeletal muscles to fatiguing stimulation [8]. We compared the contractility and fatigability of intact skeletal muscles isolated from the MG29 knockout mice with those from wild type (control) mice, and identified three phenotypes: a) an increased rate of

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fatigue development in EDL muscle, b) an increased extent of fatigue in EDL and soleus (SOL) muscles, and, c) a lesser recovery of force in EDL and SOL muscles after fatigue.

To elucidate the cellular basis for the three phenotypes in EDL muscles from *mg29* knockout mice, we investigated the effects of caffeine on the isometric contractile properties of intact muscles during the recovery from fatiguing stimulation. We also measured Ca²⁺ uptake, Ca²⁺/caffeine-induced Ca²⁺ release and total Ca²⁺ release properties of α -toxin-skinned EDL muscle fibers from control and *mg29* knockout mice. Intact EDL muscles from *mg29* knockout mice recovered significantly less than control muscles even in the presence of high concentrations of caffeine. In skinned muscle fibers, we found that submaximal Ca²⁺ uptake is slowed and Ca²⁺/caffeine-induced Ca²⁺ release is modified in such a way to suggest that it was dramatically enhanced in mutant muscles and total Ca²⁺ stored within the SR is significantly less, suggesting a leakier SR in mutant muscles.

MATERIALS AND METHODS

Generation of mutant mice

The procedure for generation of *mg29* knockout mice used in this study has been published elsewhere [7]. These mice were generated from the C57BL/6J background. For comparative studies, the SV129/J x C57BL6 strain was used as the wild-type control. Care was taken to ensure that all procedures relating to the living animals were in accordance with the "Guiding Principles in the Care and Use of Animals" approved by the American Physiological Society.

Intact muscle preparation

The detailed experimental protocol for isolation of intact muscle from mice has been described by Brotto *et al* [10]. Briefly, pairs of intact EDL muscles, 1 control, and 1 mutant, were mounted vertically on two Radnoti (Monrovia, CA) glass apparatus with platinum stimulating electrodes. The isometric contractile force of the intact muscles was monitored on a strip chart recorder, digitized and stored for later analysis. After muscles were mounted, the resting tension, current and frequency of stimulation (~ 300 mA and 100 Hz.) were adjusted to produce maximal tetanic force (i.e., T_{max}). The twitch force and T_{max} were normalized to force/cross sectional area by using the following relationship: F/cm² = [force (g)*muscle length*1.06]/muscle weight. To follow the time course and recovery from fatiguing stimulation, all force data were normalized to the T_{max} event measured just prior to the fatiguing protocol. Force versus frequency data was normalized to the maximum force generated by each muscle. Upon completion of an experimental protocol, the muscles were removed from the bathing medium and rapidly transferred to the skinning solution described below.

α -Toxin-skinned Muscle Fibers

These experiments followed the protocols described in Brotto and Nosek [11] as recently modified in Laukinobis and Stephenson [12]. Small bundles of muscle fibers (5-10 fibers), were exposed for

30 min to 5 μ M α -toxin. In this study, we utilized α -toxin-skinned muscle fibers for the SR Ca²⁺-uptake and -release experiments, instead of saponin-skinned muscle fibers, because Laukinobis and Stephenson [12] have recently demonstrated that saponin and β -escin may exert deleterious effects on the SR membrane. In agreement, we have found that caffeine responses are more robust, reproducible and consistent in α -toxin-skinned muscle fibers when compared with saponin-skinned muscle fibers. Muscle fibers were mounted between an optoelectric force transducer (Scientific Instruments GMBH, Heidelberg, Germany) and a movable arm by wrapping the fibers 3 times around small stainless steel wires. Only a very small part (~ 300-500 μ m) of the fiber remained free in between the two arms and the muscle fibers were stretched by 30% (sarcomere length ~ 2.4-2.6 μ m). The fibers were bathed in solutions contained in 2.5 ml troughs milled in a spring-loaded Plexiglas plate. Single muscles were subsequently exposed to the solutions that comprised the specific protocols for SR Ca²⁺-Uptake, Ca²⁺/caffeine-induced Ca²⁺ release and total Ca²⁺ content of the SR.

SR Ca²⁺-uptake

For all the experiments in which we tested SR function, each fiber was initially incubated in the wash solution, loaded with Ca²⁺ for 10 s and exposed to caffeine. The majority (~85-90%) of the fibers reproducibly responded to caffeine, while less than 50% responded when skinned with saponin in this pre-test phase in agreement with the findings of Laukinobis and Stephenson [12]. Muscle fibers are changed from one solution to the other within 1-2 s and are incubated in each solution as follows: first, they were equilibrated in a relaxing solution (1.0 Mg²⁺; 5.0 MgATP; 15 phosphocreatine; 140.0 KMS; 50.0 imidazole, 200 ionic strength, 5.0 EGTA, pCa < 8.5, pH 7.1), then the SR was depleted of all Ca²⁺ releasable with caffeine (1.0 Mg²⁺; 5.0 MgATP; 15 phosphocreatine; 140.0 KMS; 50.0 imidazole, 200 ionic strength, 5.0 EGTA, pCa < 8.5, pH 7.1 and 25 mM caffeine), the SR was then loaded with Ca²⁺ (1.0 Mg²⁺; 5.0 MgATP; 15 phosphocreatine; 140.0 KMS; 50.0 imidazole, 200 ionic strength, 5.0 EGTA, pCa 6.0, pH 7.1) and the amount of Ca²⁺ loaded into the SR was probed by the contracture response induced by a caffeine-induced Ca²⁺ release solution (0.1 Mg²⁺; 0.1 MgATP; 5 phosphocreatine; 140.0 KMS; 50.0 imidazole, 200 ionic strength, 0.1 EGTA, pCa < 8.5, pH 7.1, 25 mM caffeine). From these experiments, a relationship (% of SR Ca²⁺ loading (obtained from the graded caffeine contractions) vs. Loading time (in s) was obtained and from this curve, the time to load the SR by 50% was calculated.

Ca²⁺/caffeine-induced Ca²⁺ release

The initial three steps were identical to those used for the SR Ca²⁺-uptake protocol and then the muscle fibers were incubated for a fixed amount of time (30 s) in the loading solution. The fibers were then incubated for 2 min in a rigor solution (1.0 Mg²⁺; 0.0 MgATP; 140.0 KMS; 50.0 imidazole, 200 ionic strength, 1.0 EGTA, pCa < 8.5, pH 7.1) to inhibit the SERCA pump and prepare the internal milieu for the CICR phase, which comprised of a 30 s exposure to either the control (zero Ca²⁺) or the CICR solution (0.1 Mg²⁺; 0.0 MgATP; 140.0 KMS; 50.0 imidazole, 200 ionic strength, 0.2 EGTA, pCa 5.0, pH 7.1), followed by a 30 s exposure to a solution designed to stop the release of Ca²⁺ from the SR (10.0 Mg²⁺; 0.0 MgATP; 140.0 KMS; 50.0 imidazole, 200 ionic strength, 0.2 EGTA, pCa 5.0, pH 7.1). The last three steps of the CICR protocol were also identical to the last three steps used for the SR Ca²⁺-uptake protocol. Note

that each muscle fiber passes through this cycle three times. In the first run, they are exposed to pCa 8.5 solution. On the second to pCa 5.0 solution and on the third run to pCa 8.5 solution. Because pCa 8.5 solution has nominal Ca^{2+} equal to zero, no Ca^{2+} is expected to be released except any Ca^{2+} that may passively leak from the SR due to the low Mg^{2+} concentration in this solution. The caffeine responses after the two bracketed exposures of the muscle fibers to this control solution (pCa 8.5) are normalized to 100%, which means that 100% of Ca^{2+} was left within the SR. During the second run, the muscle fibers are exposed to pCa 5.0 and their response to caffeine after this exposure is decreased, indicating that significantly less Ca^{2+} was left within the SR as Ca^{2+} was released during the exposure to the pCa 5.0 solution. This difference between the average of the two bracketed responses after 8.5 and the response after pCa 5.0 is taken as Ca^{2+} /caffeine-induced Ca^{2+} release index.

Ionomycin-induced Ca^{2+} release (IICR)

In a separate series of experiments, we exposed the muscle fibers to 5 μM ionomycin after they had been exposed to caffeine. The goal was to test whether or not control and mutant muscles displayed any differences in total SR Ca^{2+} content. The pre-test phase and the initial three steps were identical to those used for the Ca^{2+} /caffeine-induced Ca^{2+} release experiments. After loading the fibers for 30 s, they were exposed to the caffeine-induced Ca^{2+} release solution. The fibers were then transferred to a relaxing solution and then exposed a solution with the composition identical to the caffeine-induced Ca^{2+} release solution, except that caffeine was replaced with 5 μM ionomycin. Ionomycin responses were normalized to the caffeine response obtained in the same fiber before exposure to ionomycin. Exposure to ionomycin rendered the muscle fibers unresponsive to further stimulation by either caffeine or ionomycin. The combination of the responses to caffeine and ionomycin was interpreted as the total Ca^{2+} stored within the SR.

Statistics

SigmaStat (Jandel Corp.) was used to statistically evaluate all data. Data are expressed as mean \pm SEM. One-way ANOVA or Kruskal-Wallis ANOVA on ranks were performed on parametric and nonparametric data, respectively, to compare differences among groups for an individual variable with a $P = 0.05$ accepted as the criterion for statistical significance.

RESULTS

Effects of Fatiguing Stimulation on Intact and Triton-Skinned Muscles

We have previously demonstrated that fast-twitch muscles of mutant mice display a phenotype characterized by increased fatigability and lesser recovery from fatigue [8]. Here, we compared the effects of fatiguing stimulation on the maximal tetanic force (T_{\max}) of intact muscles in the presence of caffeine. Fig. 1 confirms our previous findings that intact EDL muscles from mutant animals fatigue to a greater extent ($P < 0.05$) than muscles from control animals. Intact muscles from mutant animals also recover to a lesser extent ($P < 0.03$) after a 20 min recovery period. Recovery of T_{\max} is complete in the control muscles

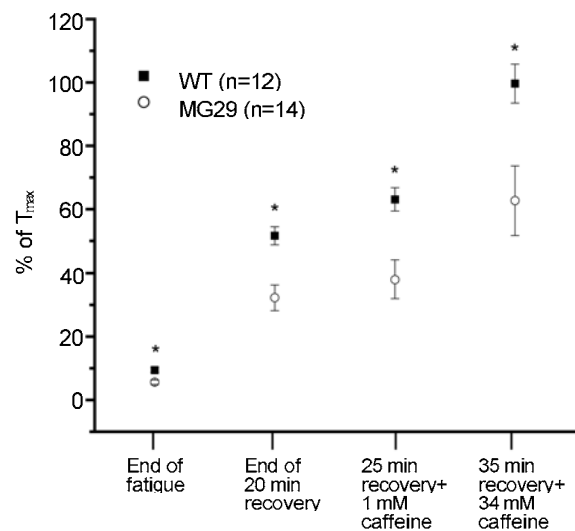


Fig. 1 Effects of fatiguing stimulation and caffeine treatment on the T_{\max} recovery of EDL muscles from WT (solid squares) and MG29 knockout muscles (open circles). * means $P < 0.05$.

only after exposure to caffeine indicating that the lack of full recovery in the control muscles is due to a deficit in the ECC process, probably due to a depression of CICR [13]. In contrast, T_{\max} of muscles from mutant animals did not ($P < 0.02$) fully recover when exposed to caffeine. Even after 30 min recovery and in the presence of 34 mM caffeine, T_{\max} is approximately 30-40% below a full recovery level in mutant muscles, suggesting defective ECC machinery.

Altered Ca^{2+} uptake into the SR in *mg29* knockout mice

The changes in membrane structure may affect the Ca^{2+} recycling properties of the mutant muscle. To examine the Ca^{2+} transport property across the SR membrane, we used the α -toxin-skinned EDL muscle preparation. Traditionally, saponin is used to permeabilize the sarcolemma in these types of experiments. However, Launikonis and Stephenson [12] have recently demonstrated that saponin may exert detrimental effects on the SR membrane. Since α -toxin has been shown to safely permeabilize smooth muscle cells and have no apparent effects on the SR membrane [14], and we have observed that caffeine responses are more robust and reproducible in α -toxin skinned fibers, we elected to use α -toxin in this study. Fig. 2 shows that time-dependent Ca^{2+} uptake into the SR membrane is significantly different between the EDL muscle from control and mutant mice. At 15 s, SR loading was 76 ± 5 of the maximum loading in the control group, but significantly less in the mutant ($58 \pm 3\%$, $P <$

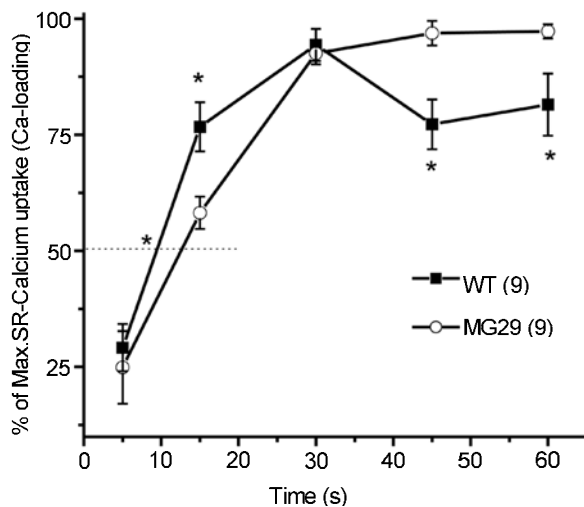


Fig. 2 SR Ca²⁺-uptake vs. time relationships in WT (solid squares) and *mg29* knockout (open circles) EDL skinned muscle fibers. Values are expressed as averages \pm SEM of the force developed in response to caffeine contractures after varied loading times (see Methods for details). All values are normalized to maximum uptake for each fiber, taken as the maximal caffeine contracture produced by each fiber. 50% loading time of the SR was achieved at significantly longer times ($*P < 0.03$) in MG29 knockout EDL muscles.

0.03). In addition, the time to achieve 50% loading of the SR was significantly longer in the mutant muscles (9 ± 1 s in control muscles vs. 13 ± 2 in mutant muscles, $P < 0.03$). Thus, the physiologically relevant, submaximal Ca²⁺ loading of the SR is significantly slower in the mutant. Notice that in both control and mutant EDL muscles loading was essentially identical at 30 s, but control muscles displayed a 15% decrease in SR loading at 45 and 60 s ($P < 0.03$) which was not observed in mutant EDL muscles. We believe that as the SR is near-maximally loaded with Ca²⁺, because CICR is functional in the control muscles, the longer exposure time to the loading solution may trigger Ca²⁺ release due to CICR from the SR.

Ca²⁺/caffeine -induced Ca²⁺ release is modified in *mg29* knockout mice

The steady-state changes in Ca²⁺/caffeine -induced Ca²⁺ release in the mutant EDL muscle were examined in the next series of studies (Fig. 3). Others and we have previously demonstrated that under experimental conditions similar to those used here, CICR is present and functional in rabbit [15], mouse [16] and rat [11] skinned muscle fibers. Endo and Takeshima [17] have also demonstrated that CICR is present and functional in skinned skeletal myocytes from mice. These authors concluded that CICR was mediated via RyR-1 and RyR-3 in mammalian skeletal

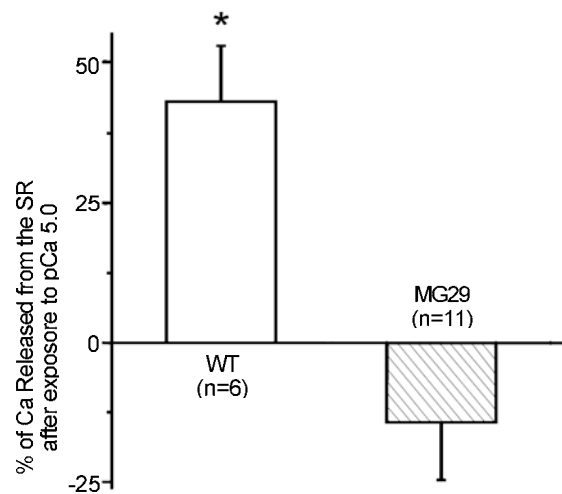


Fig. 3 Ca²⁺/caffeine -induced Ca²⁺ release properties of WT (white column) and *mg29* knockout (white/black column) EDL skinned muscle fibers. Values are expressed as means \pm SEM of the amount of Ca²⁺ released from the SR by exposure of the muscle fibers to a pCa 5.0 solution (i.e., 10 μ M free Ca²⁺) as compared to a pCa 8.5 solution (i.e., zero nominal Ca²⁺). * means $P < 0.001$.

muscles, because in permeabilized myocytes lacking both the RyRs, CICR mechanism was completely lost, and caffeine failed to induce Ca²⁺ release [15]. As no significant differences for SR loading was detected at ~ 30 s loading time for both wild type and mutant EDL muscles, this period of loading was used in these series of experiments. It is important to ensure that both groups of muscles were equally loaded, since SR loading will influence Ca²⁺ release activity. After this 30 s loading period, the SR was incubated for 30 s to two bracketed exposures of a zero Ca²⁺ solution (pCa 8.5) or a (pCa 5.0, i.e., 10 μ M free Ca²⁺) solution. The amount of Ca²⁺ remaining in the SR after exposure to these solutions was assessed by measuring the contractile force produced when the skinned fibers were exposed to a combination of a high concentration of caffeine (i.e., 25 mM) and a low concentration of magnesium (i.e., 0.1 mM). These experimental conditions are expected to promote the release of all the SR Ca²⁺ that is releasable by caffeine [18]. Furthermore, a solution containing 10 μ M free Ca²⁺ releases Ca²⁺ from the SR via the CICR process in mammalian skinned muscle fibers [11, 15, 17, 19]. Here, we found that Ca²⁺/caffeine -induced Ca²⁺ release in muscle fibers from mutant mice were significantly ($P < 0.001$) different than that displayed by muscle fibers from controls. The results of these experiments are summarized in Fig. 3.

To further characterize the effects of the mutation on Ca^{2+} handling by the SR, we compared the ability of ionomycin to release Ca^{2+} from the SR after the fibers had been treated with caffeine. Sorenson *et al* [20] using mammalian skinned muscle fibers and SR vesicles demonstrated that caffeine increases the Ca^{2+} permeability in a limited population of SR membranes and that these membranes coexisted with a population of caffeine-insensitive membrane within the same fiber. In contrast, ionomycin is a Ca^{2+} ionophore, which allows all Ca^{2+} (i.e., even the Ca^{2+} that is not releasable by caffeine) to be released from the SR [21, 22]. Muscle fibers were loaded for 30 s and then exposed to caffeine. After exposure to caffeine, muscles were allowed to relax and subsequently exposed to 5 μM ionomycin. Under these conditions the fibers from control muscles contracted to approximately $85 \pm 35\%$ of the magnitude of the contraction obtained in response to caffeine, while mutant muscles contracted to only $18 \pm 15\%$ ($P < 0.001$) (Fig. 4). These results suggest that total Ca^{2+} within the SR of muscles from mutant animals is dramatically reduced; i.e., there is defective Ca^{2+} handling in mutant EDL muscles, characterized by either a leaky Ca^{2+} pathway or an avidly Ca^{2+} -releasable pool in the SR. Another possibility is that during the relaxation phase after exposure to caffeine, Ca^{2+} was re-uptaken into the SR. Since submaximal SR Ca^{2+} uptake is slower in mutant muscles (as shown in Fig. 2), it is possible that less Ca^{2+} was available to be released by ionomycin treatment in the mutant skinned fibers as compared with control fibers. In any event, we believe that from the SR Ca^{2+} uptake, Ca^{2+} /caffeine-induced Ca^{2+} release and ionomycin experiments, it can be concluded that the SR was leakier (or less loaded with Ca^{2+}) and also that the ECC machinery is defective in mutant muscles.

DISCUSSION

Pioneering studies by Takeshima and coworkers suggested that MG29 participates both structurally and functionally in the ECC process of skeletal muscle [6, 7]. Recently, our laboratories further expanded this initial proposal by demonstrating that the absence of MG29 in fast- and slow-twitch skeletal muscles determines a phenotype characterized by an increased fatigability in these muscles [8]. In an attempt to further advance our current understanding of how MG29 is involved in the ECC process under resting and fatiguing conditions, we investigated the cellular mechanisms that might lead to the phenotypes we have identified in mice lacking the *mg29* gene. Therefore, we studied the effects of *mg29* deletion on the contractile properties of intact muscles in the presence of caffeine and the characteristic properties of both Ca^{2+} uptake and CICR of α -toxin-skinned muscle

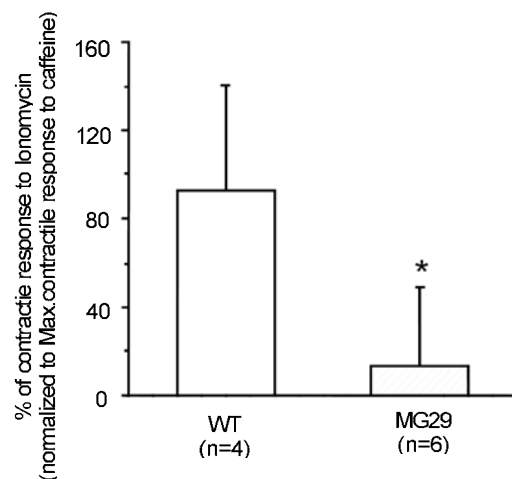


Fig. 4 Ionomycin-induced Ca^{2+} release from the SR of WT and *mg29* knockout EDL skinned-muscle fibers. Values are expressed as means \pm SEM of the magnitude of force produced by exposure of the muscle fibers to 5 μM ionomycin. Responses were normalized to the caffeine response produced by each fiber prior to exposure to ionomycin. * means $P < 0.001$.

fibers. We found that recovery from fatiguing was significantly lower in mutant EDL muscles even in the presence of high concentrations of caffeine.

Because Ca^{2+} metabolism also significantly affects force development in intact muscles, we investigated the effect of MG29 on the SR Ca^{2+} -uptake and Ca^{2+} /caffeine-induced Ca^{2+} release processes and found that EDL muscles lacking the MG29 protein display a disrupted ECC process characterized by a significantly slower Ca^{2+} uptake and an essential abolishment of CICR. A striking finding in our studies was the altered behavior of CICR in mutant EDL muscles. The seemingly loss of the steady-state response to CICR could result from two possible mechanisms: a) complete loss of Ca^{2+} sensitivity of the process, or, b) dramatically increased sensitivity to Ca^{2+} making an effect of the elevated Ca^{2+} levels used in the CICR protocol essentially ineffectual. Evidence for this latter interpretation is supported by our finding that the total Ca^{2+} pool of the SR (assessed by Ca^{2+} release in response to ionomycin after treatment with caffeine) is dramatically reduced in skinned fibers from the *mg29* knockout mice. In addition, our protocols do not allow for a continuous recording of the CICR process, but rather measures the net final response after the exposure time to either a nominal zero Ca^{2+} solution or a solution containing 10 mM free Ca^{2+} , and it may also reflect a combination of the responses to both Ca^{2+} and caffeine. Because caffeine is thought to act via a CICR-like mechanism, we conclude

that the leakage pathway in mutant muscles is associated with the increased sensitivity of CICR to myoplasmic Ca²⁺.

Therefore, the increased fatigability of mutant EDL muscles can result from a combination of a decrease in the physiologically relevant, submaximal Ca²⁺ loading of the SR, modification of Ca²⁺/caffeine-induced Ca²⁺ release, and a reduction in total SR Ca²⁺ content. Taken together, we have demonstrated that MG29 is a major player in the cascade of events that regulate and modulate normal ECC process. Disruption of the normal triad seems to lead to a subsequent disruption of the ECC process that is ultimately responsible for the increased fatigability in skeletal muscles from MG29 null mice.

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REFERENCES

- 1 Franzini-Armstrong C, Jorgensen AO. Structure and development of E-C coupling units in skeletal muscle. *Annu Rev Physiol* 1994; **56**:509-34.
- 2 Rios E, Pizarro G. Voltage sensors and calcium channels of excitation-contraction coupling. *NIPS* 1988; **3**:223-7.
- 3 Fleischer S, Inui M. Biochemistry and biophysics of excitation-contraction coupling. *Annu Rev Biophys Chem* 1989; **18**:333-64.
- 4 Suda N, Franzius D, Fleig A, et al. Ca²⁺-induced Ca²⁺ release in Chinese hamster ovary (CHO) cells co-expressing dihydropyridine and ryanodine receptors. *J Gen Physiol* 1997; **109**:619-31.
- 5 MacKrell JJ. Protein-protein interactions in intracellular Ca²⁺-release channel function. *Biochem J* 1999; **337** (Pt 3):345-61.
- 6 Takeshima H, Shimuta M, Komazaki S, et al. Mitsugumin29, a novel synaptophysin family member from the triad junction in skeletal muscle. *Biochem J* 1998; **331** (Pt 1):317-22.
- 7 Nishi M, Komazaki S, Kurebayashi N, et al. Abnormal features in skeletal muscle from mice lacking mitsugumin29. *J Cell Biol* **1999**; **147**:1473-80.
- 8 Nagaraj RY, Nosek CM, Brotto MA, et al. Increased susceptibility to fatigue of slow- and fast-twitch muscles from mice lacking the *MG29* gene. *Physiol Genomics* **2000**; **4**:43-9.
- 9 Llona I. Synaptic like microvesicles: do they participate in regulated exocytosis? *Neurochem Int* 1995; **27**:219-26.
- 10 Brotto MA, Andreatta-van Leyen S, Nosek CM, Brotto LS, Nosek TM. Hypoxia and Fatigue-induced Modification of Function and Proteins in Intact and Skinned Murine Diaphragm Muscle. *Pflugers* 2000; **440**:727-34.
- 11 Brotto MA, Nosek TM. Hydrogen peroxide disrupts Ca²⁺ release from the sarcoplasmic reticulum of rat skeletal muscle fibers. *J Appl Physiol* 1996; **81**:731-7.
- 12 Launikonis BS, Stephenson DG. Effects of beta-escin and saponin on the transverse-tubular system and sarcoplasmic reticulum membranes of rat and toad skeletal muscle. *Pflugers* 1999; **437**:955-65.
- 13 Brotto LS, Brotto MA, Nosek CM, Nosek TM. The effects of fatiguing stimulation on calcium-induced calcium release (CICR) of skinned muscle fibers. *Biophys J* 2001; **80**:379.
- 14 Kitazawa T, Kobayashi S, Horiuti K, Somlyo AV, Somlyo AP. Receptor-coupled, permeabilized smooth muscle: Role of the phosphatidylinositol cascade, G-proteins, and modulation of the contractile response to Ca²⁺. *J Biol Chem* 1989; **264**:5339-42.
- 15 Ikemoto T, Iino M, Endo M. Enhancing effect of calmodulin on Ca²⁺-induced Ca²⁺ release in the sarcoplasmic reticulum of rabbit skeletal muscle fibres. *J Physiol* 1995; **487**:573-82.
- 16 Thompson VF, Lawson K, Goll DE. Effect of mu-calpain on m-calpain. *Biochem Biophys Res Commun* 2000; **267**:495-9.
- 17 Ikemoto T, Komazaki S, Takeshima H, et al. Functional and morphological features of skeletal muscle from mutant mice lacking both type 1 and type 3 ryanodine receptors. *J Physiol* 1997; **501**:305-12.
- 18 Lamb GD, Cellini MA, Stephenson DG. Different Ca²⁺ releasing action of caffeine and depolarisation in skeletal muscle fibres of the rat. *J Physiol* 2001; **531**:715-28.
- 19 Ikemoto T, Takeshima H, Iino M, Endo M. Effect of calmodulin on Ca²⁺-induced Ca²⁺ release of skeletal muscle from mutant mice expressing either ryanodine receptor type 1 or type 3. *Pflugers Arch* 1998; **437**:43-8.
- 20 Sorenson MM, Coelho HS, Reuben JP. Caffeine inhibition of calcium accumulation by the sarcoplasmic reticulum in mammalian skinned fibers. *J Membr Biol* 1986; **90**:219-30.
- 21 Bhogal MS, Colyer J. Depletion of sarcoplasmic reticulum calcium prompts phosphorylation of phospholamban to stimulate store refilling. *Ann N Y Acad Sci* 1998; **853**:260-3.
- 22 Toth A, Ivanics T, Ruttner Z, et al. Quantitative assessment of [Ca²⁺]_i levels in rat skeletal muscle *in vivo*. *Am J Physiol* 1998; **275**:H1652-62.