Response to caffeine and ryanodine receptor isoforms in mouse skeletal muscles

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Rossi, R., R. Bottinelli, V. Sorrentino, and C. Reggiani. Response to caffeine and ryanodine receptor isoforms in mouse skeletal muscles. Am J Physiol Cell Physiol 281: C585–C594, 2001.—The response to caffeine was studied in mouse muscles [diaphragm, soleus, and extensor digitorum longus (EDL)] with different ryanodine receptor isoform (RyR1, RyR3) composition and in single permeabilized muscle fibers dissected from diaphragm of wild-type (WT) and RyR3-deficient (RyR3−/−) mice at 1, 15, 30, and 60 postnatal days (PND). The caffeine response decreased during development, and, in adult mice, was greater in diaphragm, lower in EDL, and intermediate in soleus. This suggests a direct relation between response to caffeine and RyR3 expression. The lack of RyR3 reduced caffeine response in young, but not in adult mice, and did not abolish the age-dependent variation and the intermuscle differences. In diaphragm single fibers, the response to caffeine increases during development and was reduced in fibers lacking RyR3 both at 15 and 60 PND. A population of fibers highly responsive to caffeine was present in adult WT and disappeared in RyR3−/−. The results confirm the contribution of RyR3 to calcium release for contractile response and clarify the contribution of RyR3 to developmental changes and intermuscle differences.

α-RyR and RyR1 are essential for calcium release in response to membrane depolarization. Birds affected by a spontaneous mutation producing total lack of α-RyR (16) and transgenic mice carrying null mutation of RyR1 are not vital, because calcium cannot be released and contraction triggered after membrane depolarization (28). Controversial results have been obtained about the involvement of RyR3 in the release of calcium for contraction. A decrease of tension developed in response to low-frequency stimulation and to caffeine have been found when comparing diaphragm strips from 15-day-old wild-type mice and from mice of the same age carrying a null mutation of RyR3 (3). No alterations in calcium release have been observed in RyR3-deficient myotubes during step depolarization by Dietze et al. (9). No difference has been found by Clancy et al. (6) comparing the force-frequency relations and the fatigue resistance of diaphragm strips from adult wild-type and RyR3 knockout mice. A recent study (7) has shown that, in mammalian embryonic skeletal muscle cells, calcium sparks induced by caffeine are larger and last longer in the presence of RyR3 alone than in the presence of RyR1 alone. Thus, using a completely unrelated approach, the results of Conklin and co-workers (7) are consistent with the observations of our group (3) that caffeine response is an optimal condition to identify the contribution of RyR3 to calcium release. This conclusion prompted us to further analyze the response to caffeine of muscles with distinct RyR isoform content.

It has been known for a long time that the response to caffeine varies depending on animal species, muscle type, and age. In amphibian phasic muscle fibers (sartorius, semitendinosus) the threshold for caffeine contracture lies between 1 and 2 mM, and the amplitude of a contracture induced by 10 mM caffeine can reach that of a fused tetanus (20). Determination of intracellular calcium concentrations with calcium-sensitive photoproteins has shown that concentrations as low as 0.4 mM produce an increase in intracellular calcium, which can be antagonized by specific inhibitors of calcium release.
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...such as extensor digitorum longus (EDL): from a low percent of tetanic tension at 20°C to ~20% of tetanic tension at 37°C. A slow muscle such as soleus is more responsive, since the greatest caffeine contracture can reach 50% of tetanic tension (15). The measurement of intracellular calcium with photoprotein equorin shows that the first detectable increases in intracellular calcium concentration are induced by 0.2 mM in soleus and 1 mM in EDL (13). The greater sensitivity of the slow soleus muscle compared with a fast muscle has been confirmed on glycerol-treated single fibers (24). Finally, neonatal muscles are more responsive to caffeine than adult muscles (3).

The reason for the lower sensitivity (higher threshold) and for the lower responsiveness (smaller contracture amplitude) to caffeine of mammalian compared with amphibian muscles, of fast compared with slow muscles, and of adult compared with immature muscles is not yet clear. As described by Makabe et al. (21), the amplitude of the intracellular calcium transient induced by caffeine depends on the interplay between release and removal of calcium. The difference among muscles can be therefore located either in the release mechanism or in the removal processes. In regard to the release mechanism, the correspondence between the large response to caffeine and the presence of two isoforms of RyR both in amphibian muscles and in newborn mammalian muscles and the decrease response to caffeine in RyR3-deficient mice (3) lend support to the view that the amplitude of the caffeine response might be directly related to the presence of RyR3.

In this study we aimed to further investigate the role of RyR3 in releasing calcium for contraction, with specific reference to the caffeine contracture, since in this condition the difference between wild-type and RyR3-deficient animals is best visible. We extended the study from the comparison between wild-type and RyR3 knockout animals to the comparison between 1) newborn and adult animals that have high and very low expression of RyR3 (3) and 2) three muscles, diaphragm, soleus, and EDL, where RyR1 is associated with high, intermediate, and no content of RyR3, respectively (3). We also studied the caffeine response of single muscle fibers, dissected from the diaphragm of 15- and 60-day-old mice and permeabilized with a mild treatment with saponin.

The results clearly support the view that the presence of RyR3 can amplify the amount of calcium released from SR in the presence of caffeine and that calcium released through RyR3 contributes to the activation of the contractile proteins. The presence of RyR3, however, does not explain completely the differences between fast and slow muscles and the developmental variations in caffeine response.

METHODS

Animals and Muscle Dissection

Wild-type and RyR3 knockout mice (strain C57) at birth and at the age of 15, 30, and 60 postnatal days (PND) were studied. Generation of RyR3+/− knockout mice has been previously described (3). Mice were genotyped by Southern blot and PCR assays (2).

The animals were killed by cervical dislocation. The leg and lower part of the chest including ribs and diaphragm were removed and immersed in oxygenated Krebs solution. In mice of 15, 30, and 60 PND, diaphragm strips (width 1–2 mm) from central tendon to ribs were dissected under a stereomicroscope (×10 to ×60, wild type). In mice of 1 PND, diaphragm strips included the central tendon and spanned from the right to the left rib insertion. Soleus and EDL muscles were dissected from the leg and their tendons tied with thin silk thread.

Caffeine Contracture in Intact Muscles

Experimental setup and protocol. The preparations (diaphragm strips or soleus or EDL muscles) were transferred to the myograph, where they were mounted between the hook of a force transducer (AME 801; Aksjeselkapet Mikkroelektronik, Norway) and a hook connected to a movable shaft was used to adjust muscle length. Diaphragm strips were attached by means of small holes opened between the ribs and strengthened with silk thread ligatures. Limb muscles were tied to the hooks by means of silk threads that had been previously fixed to the tendons. The perfusion bath (vol 2 ml) was filled with bicarbonate Krebs solution, bubbled with a O2-CO2 mixture kept at constant temperature (22°C). The perfusing solution could be quickly renewed (5–10 s) by flushing through the chamber ~20 ml of the new solution. On both sides of the perfusion bath at a distance of ~2 mm from the preparations, plate platinum electrodes connected with a Grass stimulator allowed field electrical stimulation.

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 ~30 min with supramaximal, low-frequency (0.1 Hz) stimuli, and then fused tetani (0.5-s duration) were recorded.

The response to caffeine in the concentration range of 5–30 mM was then tested. The solution in the chamber was quickly replaced with a new one containing caffeine at the desired concentration, and, once the contracture had fully developed, the preparation was washed with Krebs solution without caffeine to produce relaxation. In a separate group of preparations the response to 10 μM ryanodine was also tested.

Data recording and analysis. The output of the tension transducer was stored in a personal computer (PC) after analog-to-digital (A/D) conversion and was recalled for analysis. Cambridge Electronic Design (CED) 1401 A/D converter and CEA Spike2 software (CED, Cambridge, UK) were used.

Calcium Release in Single Muscle Fibers

Preparations. Single fibers were manually dissected free from the diaphragm of 15 PND (young) and 60 PND (adult) mice. Segments of ~1.5-mm length were cut and mounted with small aluminum clips (T clips) at both ends. Experimental setup. The experimental setup used was very similar to that previously described in detail (4, 5). It was composed of a force transducer (AE 801 Sensohor, Horten, Norway), an electromagnetic puller to control fiber length,
and an aluminum plate, where seven small chambers were milled. Sliding of the plate allowed quick transfer of the muscle fibers from the first, larger chamber (500 μl) to the other six, smaller chambers (70 μl) containing different solutions (skinning, relaxing, loading, washing, releasing, and activating at maximal calcium concentrations). A stereomicroscope was fitted over the apparatus to view the fiber at x20 to x60 magnification during the mounting procedure and during the experiment. The setup was placed on the stage of an inverted microscope (Axiovert 10, Zeiss, Germany). Because the floors of the muscle chambers were made by coverslips, specimens could be viewed at x320 magnification through the eyepieces of the microscope. A video camera fitted to the camera tube of the microscope and connected through an A/D converter to a computer allowed the fibers to be viewed on a monitor at x1,000 magnification and to be stored as digitized images during experiments. Signals from the force and displacement transducer were visualized on the screen of a storage oscilloscope (model 5113; Tektronix, Beaverton, OR) and on a chart recorder (WR3701; Graphitec, Japan). The signals after A/D conversion (interface 1401 plus; CED) were fed into a PC and stored in the hard disk. For data storage, recall, and analysis, the software Spike 2 (CED) was used.

Solutions. The composition of the solutions are reported in Table 1. Free ionic concentration and ionic strength of all solutions were calculated using the computer program designed by Fabiato (11).

Protocol. Fibers were transferred to the first chamber (containing solution 1) of the experimental setup and mounted between the force transducer and the electromagnetic pulser. The fibers were then moved to the first small (70 μl) cell and permeabilized with saponin (5 μg/ml for 30 s). After fibers were immersed in relaxing solution (solution 3), SR was loaded by immersing the fiber in a solution (solution 4) at pCa 6.45 in the presence of 5 mM ATP. After the fibers were washed (solution 5) to remove excess EGTA, calcium release was induced by transferring the fiber to solution 6 with low EGTA content (0.1 mM) containing caffeine in concentrations variable from 0.1 to 20 mM (pCa = 8.4). The fibers were finally transferred to a cell containing activating solution (solution 7) to measure their ability to develop force during a maximal activation (pCa = 4.7). The fibers were then brought back to relaxing solution (solution 3) and a new cycle of loading and release could be performed.

The release of calcium was detected by the transient tension development and quantified by measuring the initial rate of tension rise and tension-time area. This method, first developed by Endo and Iino (10), has been widely used (see, e.g., Refs. 14, 18, 21, and 24). The transient is generated by the combination of the calcium release from the SR with the calcium removal due to diffusion into the medium, buffering by EGTA, and reuptake in the SR (21). Experimental and model analyses (14, 21) suggest that the area gives the best indication on the amount of calcium release, whereas the rate of tension rise can be related to the rates of calcium release and of tension generation by cross-bridges. Both the rate of tension rise and the tension-time area were normalized to the tension developed during maximal activation (pCa = 4.7, solution 7) to account for the ability of the myofibrillar apparatus to develop force. After normalization, the rate of tension rise (thereafter indicated as rate) was expressed per second and the tension-time area (thereafter indicated as area) was expressed in seconds.

### Statistical Analysis

Data are expressed as means ± SE. Comparisons between wild-type and RyR3-deficient preparations were based on Student’s t-test; comparisons between different ages and different muscles were based on variance analysis followed by Newman-Keuls test. Statistical significance was set at 0.05.

Dose-response curves were interpolated using the sigmoidal curve

$$ Y = \frac{A_{\text{max}}}{1 + 10^{(\log EC_{50} - \alpha \beta)}} $$

where $A_{\text{max}}$ is the maximal response amplitude, $EC_{50}$ is the concentration at which half-maximal response is achieved, and $N$ is the Hill coefficient.

Variability analysis was carried out by fitting a Gaussian curve to distributions of the experimental measurements

$$ Y = \frac{[A/(S \sqrt{2\pi})]}{\cdot \exp \{-0.5[(x - M)/S]^2\}} $$

or a double-Gaussian curve

$$ Y = \frac{[A_1/(S_1 \sqrt{2\pi})]}{\cdot \exp \{-0.5[(x - M_1)/S_1]^2\}} + \frac{[A_2/(S_2 \sqrt{2\pi})]}{\cdot \exp \{-0.5[(x - M_2)/S_2]^2\}} $$

where $A$ and $A_1$ are areas, $M$ and $M_1$ are means, and $S$ and $S_1$ are standard deviations. Curve fitting was carried out using the software package Prism (GraphPad, San Diego, CA). The algorithm minimized the squared sum of actual distance of the experimental points from the curve. The goodness of fit was estimated using the squared sum of the distance between experimental and calculated values at the minimum.

### Table 1. Composition of solutions used in the calcium release experiments on single permeabilized fibers

<table>
<thead>
<tr>
<th>No.</th>
<th>Use</th>
<th>K-proionate</th>
<th>KH2PO4</th>
<th>DTT</th>
<th>Na-EGTA</th>
<th>Mg-acetate</th>
<th>Na2-ATP</th>
<th>Caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dissecting and mounting</td>
<td>150</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-proionate</td>
<td>Mg-proionate</td>
<td>K-EGTA</td>
<td>Ca-EGTA</td>
<td>Imidazole proprionate</td>
<td>Na2-ATP</td>
<td>Caffeine</td>
</tr>
<tr>
<td>2</td>
<td>Skinning, with saponin (5 μg/ml)</td>
<td>172</td>
<td>2.4</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Relaxing</td>
<td>172</td>
<td>2.4</td>
<td>10</td>
<td>5</td>
<td>0.1</td>
<td></td>
<td>0.1–20</td>
</tr>
<tr>
<td>4</td>
<td>Loading</td>
<td>172</td>
<td>2.4</td>
<td>10</td>
<td>5</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Washing</td>
<td>172</td>
<td>2.4</td>
<td>10</td>
<td>5</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Releasing</td>
<td>172</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Activating</td>
<td>172</td>
<td>3</td>
<td>10</td>
<td>5</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All solutions contained 1 mM sodium azide. For all solutions, pH = 7, temperature = 20°C.
RESULTS

Caffeine Contracture in Diaphragm Strips

Recordings of contractures induced by 30 mM caffeine in diaphragm strips from wild-type (RyR3+/+, A) and RyR3−/− (B) mice of different ages are shown in Fig. 1A. The differences in amplitude and in time course of the response can be appreciated from inspection of the traces. The development of tension, which was rather fast in newborn preparations (peak after 60 s), became much slower in 15-day and 30-day preparations. In these two age groups (15 and 30 days), the response was biphasic: a first peak was reached quickly and was followed by a slow prolonged tension development, which, on some occasions, did not even reach a plateau when the relaxation was induced by washing the preparation with a solution free of caffeine. In adult (60-day-old) preparations the tension development was rather fast: after

Fig. 1. Experimental traces showing the response to 30 mM caffeine of diaphragm strips from wild-type (RyR3+/+, A) and RyR3−/− (B) mice of different ages. The amplitude of the contractures is expressed as a percentage of the tension developed during a fused tetanus. Note the differences in the time scale. Arrows indicate the time when the preparation is exposed to the solution containing caffeine and the time when the preparation is washed with a caffeine-free solution to induce relaxation. The preparations were exposed to caffeine for not more than 600 s.
~10 s a peak was reached and no further slow tension rise occurred. Figure 1B shows tracings of typical caffeine contractures induced in diaphragm strips of RyR3−/− mice of the same ages (newborn, 15 days, 30 days, 60 days). As can be seen, the time course was very similar to that of wild-type preparations, but the amplitude was lower. When the contracture was induced with 10 mM caffeine, the time course of the tension development in all groups of preparations was similar to that shown in Fig. 1 for the responses to 30 mM caffeine, whereas the amplitude was lower as shown in Fig. 2.

The average values of the amplitude of the caffeine contracture expressed relative to tetanic tension are reported in the histograms of Fig. 2. The amplitude was high in newborn and decreased in 15-day-old mice preparations and even more in adult (60-day-old) preparations (see also Fig. 1). Tetanic tension was taken as a reference, since it measured the maximal ability of the myofibrillar apparatus to develop tension once maximally calcium activated. The tetanic tension developed by diaphragm strips (wild type and RyR3−/− pooled together) increased from 58 ± 13 mM/nm² in newborn mice to 104 ± 10 mM/nm² in adult mice. The twofold increase was, however, not sufficient to compensate for the six times reduction of the amplitude of the caffeine contracture. In each age group the amplitude of the caffeine contracture was lower in preparations from RyR3−/− mice than in preparations from wild-type mice. The reduction was statistically significant in newborn and in 15-day-old mice preparations.

The amplitude of the response to ryanodine in diaphragm strips of mice of different ages is shown in Fig. 2C. In the presence of 10 μM ryanodine, in all preparations examined, tension developed slowly at first, then with an increasing rate, and finally reached a plateau after 800–1,000 s. The plateau tension, expressed as percent of tetanic tension, increased from birth to adulthood. This increase likely represented an indication of the maturation of the SR and of the increased density of ryanodine receptors. No reduction of the response was observed in RyR3−/− preparations.

Comparison Among Soleus, EDL, and Diaphragm Muscles

The amplitudes of the contractures induced by caffeine in the three mouse muscles examined in this study are shown in Fig. 3. The average amplitude of the contracture is expressed relative to tetanic tension. The comparison among muscles isolated from 15-day-old mice did not show any significant difference among muscles, but showed a dramatic reduction in muscles dissected from RyR3−/− mice. In muscles isolated from adult (60-day-old) mice, the response to caffeine was 4–10 times lower than that observed in young (15-day-old) preparations. In accordance with previous studies (13, 15), the response was greater in the slow soleus than in the fast EDL, whereas the time course was faster in the EDL (peak was reached in 10–15 s) than in the soleus (time to peak 20–30 s). Surprisingly, the response of the diaphragm, which is a fast muscle, was similar to that of soleus. No significant reduction of the response was observed in muscles from RyR3−/− adult mice.

Response to Caffeine in Saponin-Permeabilized Fibers

Typical records of the tension transients induced by caffeine in a permeabilized diaphragm fiber are shown in Fig. 4. The delay between exposure to caffeine and tension development was shorter; the slope of the tension rise, the amplitude, and the area of the tension transient were clearly greater in the presence of 20 mM caffeine than in the presence of 0.5 mM caffeine. Dose-response curves of permeabilized diaphragm fibers are shown in Figs. 5 and 6. Four groups of preparations were compared: fibers from young (15-day-old) and adult (60-day-old) mice and for each age from wild-type and RyR3−/− mice. Two parameters, rate of tension rise and area of the transients, were measured to quantify the response to caffeine. As described in METHODS, both parameters were normalized to tension development during direct activation of the fiber with PCa = 4.7; this tension was considered a measure of the maximal ability to develop tension of the contractile apparatus.
The response to caffeine at the highest concentration (20 mM) showed a difference between wild-type and RyR3−/− fibers: both the area and the rate of the transients were significantly greater in wild-type than in RyR3−/− at 15 days (see Fig. 5, A and B, and Table 2). The difference in area was still present in adult mouse fibers (see Fig. 6, A and B, and Table 2). The concentrations at which one-half of the maximal response was achieved (EC50, see Table 2) did not change when RyR3 was not present and the dose-response curves normalized to the maximal response (see Fig. 5, C and D, and Fig. 6, C and D) virtually superimposed. Clear age-dependent changes of both EC50 and amplitude occurred during maturation (Figs. 5 and 6 and Table 2). The amplitude of the maximal response, as measured from tension-time area increased during maturation (compare Fig. 5, A and B, with Fig. 6, A and B), whereas a decrease of the Hill coefficient (N) in the presence of a virtually unchanged EC50 indicated a decrease of the threshold for caffeine response during maturation (compare Figs. 5 and 6 and see Table 2).

Distribution of the Amplitudes of the Maximal Caffeine Contracture

The histograms of Fig. 7 show the distributions of the maximal responses to caffeine (caffeine concentration = 20 mM) in diaphragm fibers from adult wild-type and RyR3−/− mice. The interpolation with Gaussian curves showed that the fitting was significantly improved using a double instead of a single Gaussian curve (the cost function calculated as the sum of the squares of the differences decreased from 32 to 7.5) in wild-type preparations. No significant improvement (the cost function decreased from 37 to 33) occurred in RyR3−/− mice. These results point to a bimodal distribution of the caffeine responses in wild-type fibers and to a unimodal distribution in fibers from RyR3−/− mice. In wild-type preparations, a group of fibers were able to produce larger responses to caffeine, and this group disappeared in RyR3−/− mice. No indications of bimodal distribution were detected in the maximal response to caffeine of fibers from wild-type and from RyR3−/− mice of 15 PND (data not shown).
DISCUSSION

The relevance of the type 3 ryanodine receptor (RyR3) for release of calcium in relation to contractile response is still controversial. The generation of mice lacking RyR3 has provided a powerful tool to answer the question. Experiments on neonatal mouse diaphragm strips (3) have given evidence in favor of a contribution of RyR3 to calcium release in contraction, and spark analysis of cultured embryonic muscle cells has shown a contribution of RyR3 similar to that of RyR1 (7). No effects of the deficiency of RyR3 have been detected by Dietze et al. (9) in step-depolarized myotubes and by Clancy and co-workers (6) on electrically stimulated adult diaphragm strips.

In this study we aimed to contribute to the controversial issue by extending our previous experiments on

Fig. 5. Dose-response curves of diaphragm fibers of young (15 PND) mice exposed to caffeine. Solid lines and ●, wild-type mice; dashed lines and ○, RyR3 −/− mice. A and B: absolute values of rate of tension rise and tension-time area of the transients; both parameters were normalized to tension developed during direct calcium activation (pCa = 4.7). C and D: values of rate of tension rise and tension time area normalized to the values recorded during the response to a maximal caffeine concentration (20 mM). Sigmoidal curves were fitted to the experimental points as described in METHODS, and the parameters are reported in Table 2.

Fig. 6. Dose-response curves of diaphragm fibers of adult (60 PND) mice exposed to caffeine. Solid lines and ●, wild-type mice; dashed lines and ○, RyR3 −/− mice. A and B: absolute values of rate of tension rise and tension-time area of the transients; both parameters are normalized to tension developed during direct calcium activation (pCa = 4.7). C and D: values of rate of tension rise and tension time area normalized to values recorded during the response to a maximal caffeine concentration (20 mM). Sigmoidal curves were fitted to the experimental points as described in METHODS, and the parameters are reported in Table 2.
Caffeine contracture in intact diaphragm strips is clearly peculiar changes in the kinetics of tension development. The age-related decline of the response is associated with this parameter, whereas the amplitude of the response, determined by RyR3 deficiency, shows that the effect of RyR3 deficiency is significant only in the young age groups. More careful observations have shown that the effect of RyR3 deficiency in soleus is more responsive to caffeine than EDL as previously documented (13, 15). In adult mice RyR3 is expressed in soleus but not in EDL: the lack of RyR3 in RyR3−/− mice, however, does not abolish the difference between the two muscles. Some diversity might exist in the function or in the structure of RyR1 in slow and fast fibers, as recently shown for fish muscles (22). Both in diaphragm and in soleus at 60 PND, the lack of RyR3 does not seem to impair significantly the ability to release calcium in caffeine contracture and also with electrical stimulation (3, 6). It seems that either RyR3 is no longer required or that its absence has found a compensatory mechanism.

In accordance with previous observations (3), the amplitude of the response to caffeine of diaphragm strips decreases with aging, and the effect of RyR3 deficiency is significant only in the young age groups and not in adult mice. Previous work (3) has shown that RyR3 is abundant in diaphragm during the first 3 wk after birth and much less abundant in adult (60 PND) diaphragm. This forms the basis of our view (3) that RyR3 is required to amplify the calcium release that determines the caffeine contracture. Following this reasoning, the decrease in amplitude related to skeletal muscle maturation might be attributed to the age-related disappearance of RyR3. More careful observations, however, show that the effect of RyR3 deficiency is just on the amplitude of the response, whereas the age-related decline of the response is associated with peculiar changes in the kinetics of tension development. The kinetics of tension development during caffeine contracture in intact diaphragm strips is clearly monophasic at birth, then becomes biphasic, and the second phase becomes progressively smaller with age. The amplitude of the second phase is dramatically reduced when RyR3 is lacking, but the age-dependent changes in kinetics are not modified.

In this study the comparison between wild type and RyR3−/− in the response to caffeine has been extended to soleus and EDL. In these muscles the lack of RyR3 determines a lower response to caffeine at 15 PND: at this age all muscles express significant amounts of RyR3 (3). In adult mice the difference between wild type and RyR3−/− is not significant, and the soleus is more responsive to caffeine than EDL as previously documented (13, 15). In adult mice RyR3 is expressed in soleus but not in EDL: the lack of RyR3 in RyR3−/− mice, however, does not abolish the difference between the two muscles. Some diversity might exist in the function or in the structure of RyR1 in slow and fast fibers, as recently shown for fish muscles (22). Both in diaphragm and in soleus at 60 PND, the lack of RyR3 does not seem to impair significantly the ability to release calcium in caffeine contracture and also with electrical stimulation (3, 6). It seems that either RyR3 is no longer required or that its absence has found a compensatory mechanism.
A novel result of this study was the demonstration of a difference in the response to caffeine of single fibers dissected from the diaphragm of wild-type and RyR3−/− mice. The fibers were permeabilized by short immersion in a medium containing saponin. Saponin interacts with membrane cholesterol and causes perforation of sarcolemma and transverse tubules, which are rich in cholesterol, but not of SR (14, 18). The rate of tension rise and used in studies on calcium release in permeabilized preparations (14, 18) to avoid any leakage of calcium from SR. The response to caffeine was measured from the transient tension development according to a method first described by Endo and Iino (10) and frequently used in studies on calcium release in permeabilized preparations (14, 18, 24). The rate of tension rise and the area of the tension transient were used to quantify the amplitude of the response.

The threshold for caffeine and the EC50 value were found to be lower in adult than in young preparations. Their values (in the range 0.1–1 mM) were comparable with those of caffeine-induced calcium release in isolated vesicles (see for example Ref. 19). The age-dependent changes might be related to the development and maturation of the SR. No difference in threshold or in EC50 values were found between RyR3 knockout and wild-type preparations. A significant difference was present in the amplitude of the response. Surprisingly, the difference was detectable not only in young mouse fibers but also, restricted to area, in 60 PND mouse fibers.

The reduced response to caffeine of single fibers from diaphragm of 15 PND RyR3−/− mice likely finds the same explanation proposed for the intact diaphragm strips: RyR3 has a specific ability in amplifying calcium release (3, 26) or has a specific sensitivity to caffeine (7). Several reasons can explain why, at 60 PND (adult mice), the consequence of the lack of RyR3 is visible in single fibers and not in intact diaphragm strips. A first possibility is that the sensitivity of the experiments carried out on single fibers is higher, and smaller differences can be detected. A second possibility is the intrinsic diversity of the caffeine response in the two preparations: the time course of the caffeine contracture points to a long-lasting increase in intracellular calcium in diaphragm strips where sarcolemma is intact and to a transient local increase of calcium, followed by a quick diffusion out of the permeabilized fibers. A third speculative but very intriguing hypothesis points to the presence of a soluble factor that might be responsible for the compensation of the lack of RyR3 in intact preparations. This soluble factor might be washed away once the sarcolemma is permeabilized in saponin-treated fibers.

A potentially interesting insight in calcium release properties of individual diaphragm fibers was given by the analysis of the variability of the response to maximal caffeine concentrations. The interpolation of Gaussian function suggests the existence of two populations of fibers in wild-type preparations: a smaller group of high responders to caffeine and a larger group of low responders. The group of high responders disappears in RyR3-deficient mice. The possibility of the existence of two groups of preparations recalls the heterogeneous distribution of RyR3 in adult mouse diaphragm described by Flucher et al. (12). Unfortunately, the tiny amount of RyR3 present in one fiber does not allow demonstration, with the use of Western blotting, of whether high-responder fibers have a peculiar RyR3 isoform composition.

In conclusion, this study confirms the role of RyR3 in contributing calcium for activation of the contractile apparatus. The results also define the partial contribution of RyR3 to the diversity between muscles and the age dependence of the response to caffeine. It remains still unsettled why some muscles, the diaphragm in the first place, need a second system of calcium release parallel to that based on RyR1.

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