Calcium regulation by skeletal muscle membranes of horses with recurrent exertional rhabdomyolysis

Tara L. Ward, BA; Stephanie J. Valberg, DVM, PhD; Esther M. Gallant, PhD; James R. Mickelson, PhD

Objective—To determine whether an alteration in calcium regulation by skeletal muscle sarcoplasmic reticulum, similar to known defects that cause malignant hyperthermia (MH), could be identified in membrane vesicles isolated from the muscles of Thoroughbreds with recurrent exertional rhabdomyolysis (RER).

Sample Population—Muscle biopsy specimens from 6 Thoroughbreds with RER and 6 healthy (control) horses.

Procedures—RER was diagnosed on the basis of a history of > 3 episodes of exertional rhabdomyolysis confirmed by increases in serum creatine kinase (CK) activity. Skeletal muscle membrane vesicles, prepared by differential centrifugation of muscle tissue homogenates obtained from the horses, were characterized for sarcoplasmic reticulum (SR) activities, including the Ca^{2+} release rate for the ryanodine receptor-Ca^{2+} release channel, [^{3}H]ryanodine binding activities, and rate of SR Ca^{2+}-ATPase activity and its activation by Ca^{2+}.

Results—Time course of SR Ca^{2+}-induced Ca^{2+} release and [^{3}H]ryanodine binding to the ryanodine receptor after incubation with varying concentrations of ryanodine, caffeine, and ionized calcium did not differ between muscle membranes obtained from control and RER horses. Furthermore, the maximal rate of SR Ca^{2+}-ATPase activity and its affinity for Ca^{2+} did not differ between muscle membranes from control horses and horses with RER.

Conclusions and Clinical Relevance—Despite clinical and physiologic similarities between RER and MH, we concluded that RER in Thoroughbreds does not resemble the SR ryanodine receptor defect responsible for MH and may represent a novel defect in muscle excitation-contraction coupling, calcium regulation, or contractility. (*Am J Vet Res* 2000;61:242–247)

Exertional rhabdomyolysis is a common cause of poor performance in many breeds of horses.[1] In Thoroughbred racehorses, **recurrent exertional rhabdomyolysis (RER)** affects approximately 5% of the population,[2] is most common in young fillies,[2] and is associated with various forms of stress.[1,3] There also is an increased incidence of myopathy in Thoroughbreds with RER after halothane anesthesia.[4] Rhabdomyolysis associated with stress, exercise, or anesthesia also is observed in disorders such as **malignant hyperthermia (MH)** in humans and pigs, which is attributed to abnormalities of calcium regulation and excitation-contraction coupling in skeletal muscles.[5,6] Studies have documented significantly lower thresholds for potassium-, caffeine-, and halothane-induced contractures in intact skeletal muscle fiber bundles isolated from Thoroughbreds with RER, compared with healthy control horses.[7] Similar alterations in the sensitivity of muscle biopsy specimens to pharmacologically-induced contracture are diagnostic for MH susceptibility in humans and pigs, indicating a possible similarity between RER in Thoroughbreds and MH in other species.[8-10] Another report indicates that muscle fibers from horses of unspecified breeds with exertional rhabdomyolysis have a profound increase in resting myoplasmic Ca^{2+} concentration, as determined by use of Ca^{2+} microelectrodes.[11] A similar result is observed for muscle fibers from MH-susceptible humans.[12]

Malignant hyperthermia and MH-like disorders can arise from a number of genetic defects that affect regulation of excitation-contraction coupling and Ca^{2+} regulation in skeletal muscle.[5,13] Our laboratory group has documented that RER in many families of Thoroughbreds is a genetic defect inherited as an autosomal dominant gene with variable expression, which can be traced back 5 to 7 generations to a common ancestor.[14] We hypothesized that an alteration in one of the muscle proteins responsible for myoplasmic calcium regulation could be associated with this inherited form of RER. Genetic defects in MH and related inherited neuromuscular disorders include mutations in the **ryanodine receptor (RyR)** in the **sarcoplasmic reticulum (SR)**,[5,13,15] which serves as the Ca^{2+} release channel for the SR,[16] the transverse tubule dihydropyridine receptor,[17,18] which serves as the surface membrane voltage sensor, and SR Ca^{2+}-ATPase,[19,20] which serves to resequester Ca^{2+} into the SR, enabling relaxation. To test our hypothesis, we isolated preparations of skeletal muscle membranes from control horses and a family of Thoroughbreds with RER. The SR RyR was examined, using techniques that were particularly useful in detecting functional changes in this protein that result in susceptibility to MH in pigs[21,22] and humans.[23] Additionally, we examined functional properties of SR Ca^{2+}-ATPase in these isolated membrane preparations.

Materials and Methods

Horses—Six Thoroughbred mares were identified with RER, which was diagnosed on the basis of a history of > 3 episodes of exertional rhabdomyolysis. Diagnosis was con-

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From the Departments of Veterinary Pathobiology (Ward, Gallant, Mickelson) and Clinical and Population Sciences (Valberg), College of Veterinary Medicine, University of Minnesota, St Paul, MN 55108.
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Address correspondence to Dr. Mickelson.

firmed by increases in serum **creatine kinase (CK)** activity (> 1,000 U/L). Four of the horses, 2 to 3 years old, had so many episodes of RER that they were unable to race. The other 2 horses (7 and 12 years old), which had performed poorly during races, were sold for use as pleasure horses and intermittently developed rhabdomyolysis. All 6 horses were related to 1 Thoroughbred stallion within the preceding 5 to 7 generations.[14] Three of the 6 horses had been tested and had positive results during halothane- and caffeine-induced muscle contracture tests.[7] Percutaneous gluteus medius muscle biopsy specimens obtained from all horses with RER contained many centrally located nuclei in type 2A and 2B muscle fibers but had normal staining for glycogen.

Control horses were selected from sedentary horses in a veterinary medical teaching herd. A dam and her 2-year-old offspring as well as 4 horses believed to be unrelated to those 2 horses were used in the study. They comprised 5 healthy Thoroughbreds and 1 Quarter Horse (3 mares, 3 geldings) ranging from 2 to 19 years old (mean, 7.3). It was known that 3 horses did not have a history of RER, whereas the exercise history for the other 3 horses could not be obtained. Examination after hematoxylin and eosin as well as periodic acid-Schiff staining of the semimembranosus muscle of these horses did not reveal histopathologic abnormalities. After completion of the experiments reported here, potassium contracture testing was performed on intercostal muscle biopsy specimens obtained from 3 of the Thoroughbreds. The threshold potassium concentration for inducing contracture in these control muscle samples was similar to that for other control horses examined but significantly different from that of Thoroughbreds with RER.[7]

Isolation of muscle membranes—Muscle biopsy specimens were obtained from the semimembranosus muscle, using lidocaine hydrochloride administered as a local anesthetic. Samples were cut into small pieces, immediately frozen in liquid nitrogen, and stored at –80 C until use. Approximately 1.5 g of muscle from each normal horse or horse with RER was homogenized and prepared as described elsewhere.[24] Briefly, differential centrifugation of the muscle homogenate prepared after addition of a cocktail of protease inhibitors, followed by extraction with 0.6*M* KCl, allowed isolation of a high yield of a membrane fraction containing SR and transverse-tubule membranes. Membrane pellets were resuspended in a solution containing 10% sucrose, 20 m*M* tris, 0.1 m*M* phenylmethlysulfonyl flouride,[a] and 0.8 m*M* benzamidine (pH 7.4). Protein concentrations were determined in duplicate, using the method of Lowry et al.[25]

Release of calcium ions from sarcoplasmic reticulum—Release of **calcium ions (Ca^{2+})** from SR vesicles was performed as described elsewhere.[22] Briefly, $^{45}Ca^{2+}$[b] was passively loaded into 0.4 mg of SR and incubated for 2 hours at 37 C in media containing 150 m*M* potassium propionate, 15 m*M* piperizine-N,N'-bis[2-ethanisulfonic acid]; **Pipes**[a] (pH 7.0), 1 μ*M* calmodulin,[a] 5 m*M* ^{45}calcium acetate$_2$ (approx 20,000 counts/min/nmol), 1 μg of aprotinin/ml, and 1 μg of leupeptin/ml. Calcium release was initiated by vortexing 2 μl of $^{45}Ca^{2+}$-loaded SR into 200 μl of release media, which contained 150 m*M* potassium propionate, 15 m*M* Pipes (pH 7.0), 8.6 m*M* **ethylene glycol-bis (β-aminoethly ether) N,N,N',N'-tetraacetic acid (EGTA)**, 2 m*M* calcium acetate$_2$ (free Ca^{2+} concentration, approx 100 n*M*), 1 μ*M* calmodulin, 3 m*M* $MgCl_2$ (free Mg^{2+} concentration, approx 0.45 m*M*), and 3 m*M* adenylyl-(β,γ-methylene) diphosphonate.[c] Release of Ca^{2+} from the SR was stopped at specific times (1 to 10 seconds, determined by use of a metronome), using a quench solution containing 150 m*M* potassium propionate, 15 m*M* Pipes (pH 7.0), 5 m*M* $MgCl_2$, 10 m*M* EGTA, and 20 μ*M* ruthenium red, followed by immediate filtration onto a 0.45-μm-membrane.[d] Radioactivity reflecting $^{45}Ca^{2+}$ retained by the SR vesicles was determined by liquid scintillation counting. Measurements at each time point were performed in duplicate experiments on membrane preparations from samples obtained from horses with RER and 4 control horses. Amount of time required for vesicles to release half of their $^{45}Ca^{2+}$ content was calculated, using the following equation:

$$R = R_{max} \times t/(t_{1/2} + t)$$

where R is amount of calcium released, R_{max} is the maximal amount of calcium released, and t is time.

Ryanodine binding assay—Ryanodine binding assays were performed for 90 minutes at 37 C, at which time the equilibrium binding was attained.[21,22] All assays contained 100 m*M* KCl, 10 m*M* Pipes (pH 7.0), a calcium acetate$_2$-EGTA buffer designed to provide the desired ionized Ca^{2+} concentration (as determined by the method of Brooks and Story[26]), 10 n*M* [^{3}H]ryanodine,[b] and 100 n*M* total ryanodine. Varying concentrations of unlabeled ryanodine, caffeine, and Ca^{2+} were added to the incubation media. Unbound ryanodine was separated from protein-bound ryanodine by filtration onto filters,[c] using a cell harvester.[f] Filters were washed with cold 100 m*M* KCl, and radioactivity was analyzed by liquid scintillation counting. Nonspecific binding was determined in tubes containing 100 μ*M* ryanodine, and these values were subtracted from the total binding to determine specific [^{3}H]ryanodine binding. Measurements for each concentration of ryanodine or caffeine were performed in duplicate experiments on membrane preparations from samples obtained from 4 horses with RER and 4 control horses. Measurements for each Ca^{2+} concentration were performed in duplicate on membrane preparations obtained from 3 horses with RER and 3 control horses.

Ca^{2+}-ATPase activity of sarcoplasmic reticulum—The ATPase activity was measured in 0.1*M* KCl, 10 m*M* Pipes (pH 7.0), 10 μ*M* A23187, and 10 μg of crude membranes at 22 C.[21] Desired Ca^{2+} concentrations in the range of 0.03 to 100 μ*M* (as determined by the method of Brooks and Story[26]) were achieved by use of calcium acetate$_2$-EGTA buffers. Reactions were initiated by the addition of 5 m*M* MgATP. Activity was expressed as micromoles of phosphorous released per minute per milligram of protein and defined as the difference between phosphorous liberation after addition of specified Ca^{2+} concentrations and after addition of 1 m*M* EGTA. Measurements for each Ca^{2+} concentration were performed in duplicate experiments on membrane preparations obtained from 4 horses with RER and 4 control horses.

Statistical analyses—All values were reported as mean ± SD. Comparisons between groups were made, using an unpaired Student *t*-test for normally distributed data. A value of $P < 0.05$ was considered significant.

Results

Calcium release from SR vesicles—Membrane vesicles in skeletal muscle samples from horses with RER and healthy horses were passively loaded with $^{45}Ca^{2+}$, and the rate of Ca^{2+} efflux was determined after rapid dilution in a chemically defined calcium release medium designed to mimic myoplasmic conditions of pH, ionic strength, and ionized Ca^{2+}, Mg^{2+}, adenine nucleotides, and calmodulin concentrations.[22] Calcium release mediated by RyR from RER and control horses was reflected by a decrease in the amount of calcium retained by the SR as a function of time (**Fig 1**). Curves depicting the mean time course of calcium efflux from

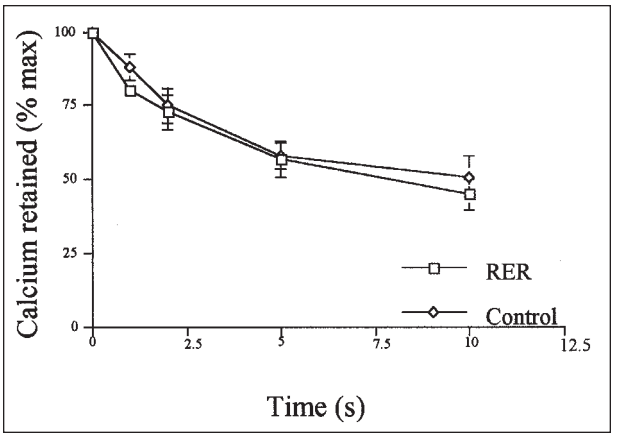

Figure 1—Calcium retention in crude skeletal muscle sarcoplasmic reticulum (SR) vesicles isolated from healthy (control) horses and horses with recurrent exertional rhabdomyolysis (RER). Vesicles were loaded with $^{45}Ca^{2+}$, and calcium retention was measured at several times after dilution in calcium release media. Amount of calcium retained is expressed as a percentage of the maximum amount of $^{45}Ca^{2+}$ retained at time 0 (% max). Values represent mean ± SD of duplicate determinations on samples obtained from 4 control horses and 4 horses with RER.

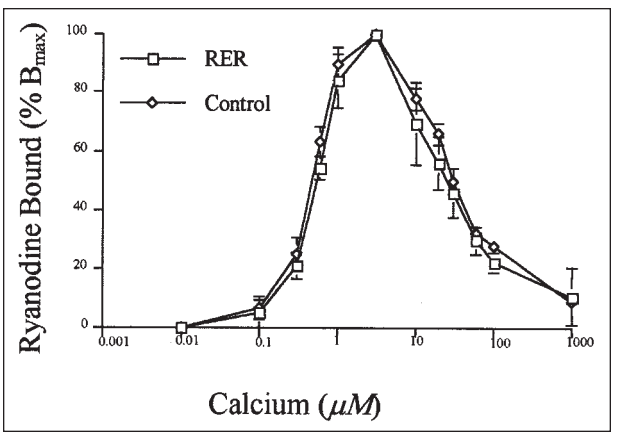

Figure 2—Effect of calcium ion (Ca^{2+}) concentration on [^{3}H]ryanodine binding to crude skeletal muscle SR vesicles isolated from control horses and horses with RER. Amount of ryanodine bound is expressed as a percentage of maximal amount of ligand bound (%B_{max}). Values represent mean ± SD of duplicate preparations obtained from 3 control horses and 3 horses with RER.

the SR were superimposable for preparations for control horses and horses with RER. The time at which SR vesicles had released half of their calcium stores was 6.1 ± 1.5 seconds for horses with RER and 7.1 ± 3.5 seconds for control horses. There was not a significant difference in the rate of calcium release between the control horses and horses with RER.

Ryanodine binding activity of SR—Calcium dependence of [^{3}H]ryanodine binding to SR vesicles of equine muscle had an expected bell-shaped curve (**Fig 2**).[5,16,21] There was not a significant difference in mean [^{3}H]ryanodine binding (adjusted on the basis of each preparation's maximal binding) between the control horses and horses with RER at any Ca^{2+} concentration. The Ca^{2+} concentration for half-maximal activation of ryanodine binding for membranes from control horses and horses with RER was approximately 0.6 µ*M*, optimal ryanodine binding was approximately 3 µ*M*, and half-maximal inhibition was approximately 30 µ*M*.

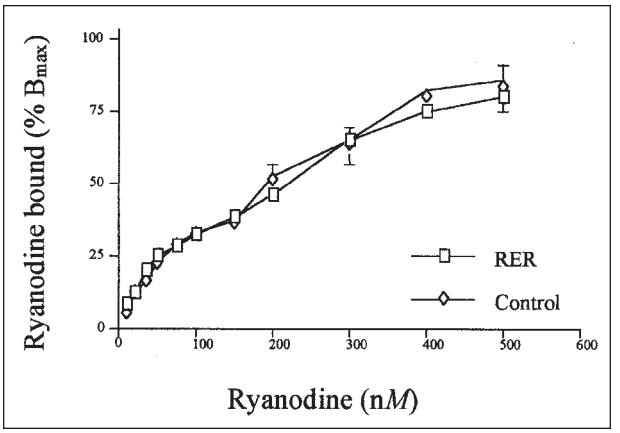

Figure 3—Effect of ryanodine concentration on [^{3}H]ryanodine binding to crude skeletal muscle SR vesicles isolated from control horses and horses with RER. Values represent percentage of the B_{max} ± SD of duplicate samples for 4 control horses and 4 horses with RER.

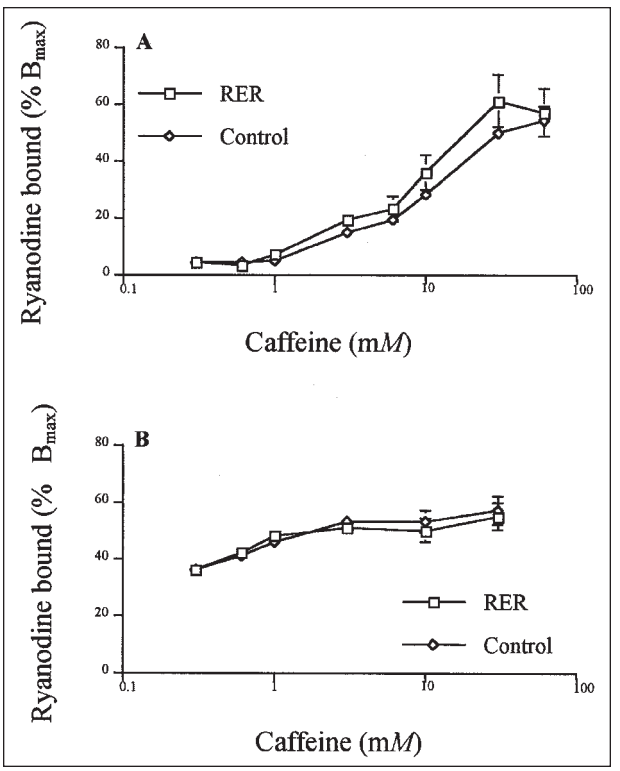

Figure 4—Effect of caffeine on [^{3}H]ryanodine binding to crude skeletal muscle SR vesicles isolated from control horses and horses with RER, using 0.1 µ*M* Ca^{2+} (A) and 3.0 µ*M* Ca^{2+} (B). Values represent percentage of B_{max} ± SD of duplicate samples for 4 control horses and 4 horses with RER.

The RyR [^{3}H]ryanodine binding curves for membranes from control horses and horses with RER after the addition of the optimal Ca^{2+} concentration of 3 µ*M* were determined (**Fig 3**). The [^{3}H]ryanodine binding increased, as expected, with increasing ryanodine concentrations for the entire range of concentrations used (ie, 10 to 500 n*M*). Using Scatchard analysis, data were used to calculate the **disassociation constant of the lig-**

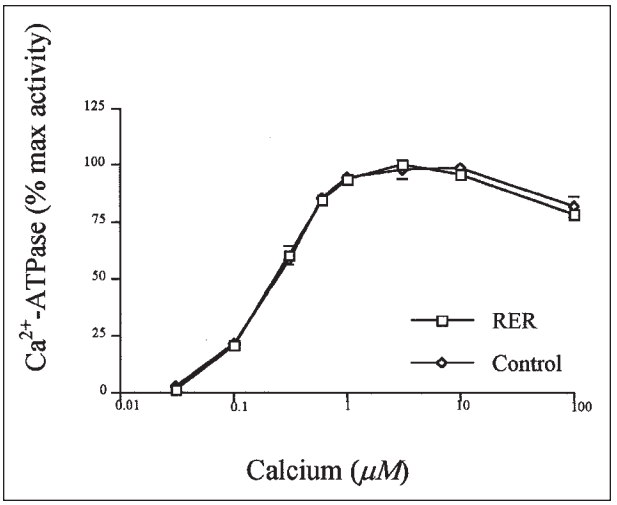

Figure 5—Effect of Ca^{2+} concentration on Ca^{2+}-ATPase activity in crude skeletal muscle SR vesicles isolated from control horses and horses with RER. Values represent percentage of maximum activity (% max activity) ± SD for duplicate samples from 4 control horses and 4 horses with RER.

and for the receptor (K_d) and the **maximal amount of ligand bound (B_{max})** for each membrane preparation. Mean K_d values for [^{3}H]ryanodine binding to the RyR of control horses and horses with RER were 197 ± 25 and 187 ± 57 n*M*, respectively, and B_{max} values were 4.0 ± 1.0 and 3.6 ± 0.9 pmol/mg for control horses and horses with RER, respectively. There was not a significant difference in the K_d or B_{max} of [^{3}H]ryanodine binding between control horses and horses with RER.

Caffeine stimulation of [^{3}H]ryanodine binding to the RyR was determined at 2 Ca^{2+} concentrations: 0.1 μ*M* Ca^{2+} to mimic resting myoplasmic Ca^{2+} concentrations (**Fig 4A**) and 3 μ*M* Ca^{2+} to mimic myoplasmic Ca^{2+} concentrations during muscle contraction (**Fig 4B**). As expected from the Ca^{2+} dependence of [^{3}H]ryanodine binding defined previously, basal amounts of [^{3}H]ryanodine binding were greater at 3 μ*M* Ca^{2+} (30% of B_{max}) than at 0.1 μ*M* Ca^{2+} (5% of B_{max}). An increase in caffeine concentrations caused an increase in [^{3}H]ryanodine binding to preparations from control horses and horses with RER at both Ca^{2+} concentrations; this stimulatory effect of caffeine was greater at 0.1 μ*M* Ca^{2+} than at 3 μ*M* Ca^{2+}. However, adjusted values for [^{3}H]ryanodine binding of membranes from control horses and horses with RER were not significantly different at any caffeine concentration, indicating a similar sensitivity to caffeine.

Calcium-ATPase activity—The Ca^{2+} dependence and maximal rate of Ca^{2+}-ATPase activity of the SR were determined for membranes from control horses and horses with RER. Adjusted curves depicting the Ca^{2+} dependence of ATPase activity were determined for both types of horses (**Fig 5**), and, using Scatchard-type analysis, data from each horse were used to calculate the **mean disassociation constant for the enzyme-substrate complex (K_m)**. The K_m for Ca^{2+} for the Ca^{2+}-ATPase of control horses and horses with RER was 0.47 ± 0.20 and 0.44 ± 0.16 μ*M*, respectively. Maximal rate of ATPase activity (obtained at 3 μ*M* Ca^{2+}) for control horses and horses with RER was 0.71 ± 0.42 and 0.68 ± 0.43 μmol of phosphorous/mg of protein/min, respectively. There was not a significant difference in K_m for Ca^{2+} or maximal rate of Ca^{2+}-ATPase activity between control horses and horses with RER.

Discussion

A study of the contractile properties of cut-fiber muscle bundles from horses with RER indicated a number of potential abnormalities for calcium regulation in skeletal muscle.[27] This included the possibilities of a shorter duration for relaxation from a caffeine contracture and a longer electrically-elicited twitch response. An increased resting myoplasmic Ca^{2+} concentration in muscle cells from horses with exertional rhabdomyolysis at 24 and 72 hours after an episode has been reported.[11] More recently, specific studies of mechanical responses of intact muscle fiber bundles obtained from a family of Thoroughbreds with RER revealed an increased rate of relaxation from twitches and tetany and an increased sensitivity of muscles to potassium-, caffeine-, and halothane-induced contractures, when compared with those for healthy control horses.[7] A biochemical or molecular basis has not been determined for these alterations in muscle samples obtained from horses with RER. The increased sensitivity of muscle samples from horses with RER to contractures induced by potassium, caffeine, and halothane is similar to that seen in animals with MH,[8-10] an inherited disorder in myoplasmic calcium regulation and excitation-contraction coupling[5] for which the most common genetic basis is mutations in the skeletal muscle RyR gene.[13,15] Malignant hyperthermia and RER also have much in common clinically, including an association with halothane exposure,[4,6] episodic onset, correlation to stress, signs of muscle cramping and pain, and destruction of muscle tissue, as evidenced by an increase in muscle enzymes and on the basis of histopathologic changes in muscle.[1,6] In another study by our laboratory group,[14] we documented that the RER trait in Thoroughbreds is inherited in an autosomal dominant fashion, suggesting that the RyR or a related gene involved in regulation of Ca^{2+} concentrations in muscle could be responsible for RER. However, the major conclusion from our studies is that, despite similarities between RER and MH, we were unable to detect an MH-like biochemical abnormality in the RyR in isolated skeletal muscle membranes from Thoroughbreds with RER.

The SR is the primary source of calcium used to initiate contractile activity in skeletal muscle. Release of Ca^{2+} from the SR is stimulated after depolarization of the surface membrane and transverse tubules (via an action potential or an increase in extracellular potassium concentration), whereas caffeine and halothane act directly on the RyR to increase Ca^{2+} channel activity.[16,28] In similar or identical biochemical assays to those performed in the study reported here, with similar numbers of animals, we documented that muscle membranes isolated from MH-susceptible pigs with the Arg615Cys RYR1 mutation have an increased rate of Ca^{2+} release from the SR,[21,22] abnormal calcium dependence curve of [^{3}H]ryanodine binding,[21] substantially

increased affinity for ryanodine,[21,22] and increased sensitivity of the SR RyR to stimulation by caffeine.[22,29] However, examination of the rate of calcium release from muscle of control horses and horses with RER did not reveal a difference in the kinetics of calcium release (Fig 1). Furthermore, we were also unable to detect an alteration in any aspect of [^{3}H]ryanodine binding between control horses and horses with RER when samples were incubated with various concentrations of Ca^{2+}, ryanodine, and caffeine (Figs 2 to 4). Therefore, analysis of our data suggests that the molecular cause of RER in Thoroughbreds probably differs from that of MH in pigs as well as most humans and probably is not caused by a defect in the RyR.

Abnormal in vitro and in vivo characteristics of skeletal muscle contracture for muscle biopsy specimens obtained from Thoroughbreds with RER could conceivably result from impaired relaxation attributable to an alteration of Ca^{2+}-ATPase content or activity in the SR. Mutations in the gene for this Ca^{2+} pump in humans are believed to be responsible for the slower rate of relaxation associated with exercise in people with Brody's disease.[19,20] However, we were unable to detect differences in Ca^{2+}-ATPase activity in SR between control horses and horses with RER (Fig 5). Furthermore, although analysis of data from another group indicated the possibility of a decreased rate of relaxation in muscle obtained from animals with RER,[27] data for intact muscle fiber bundles in our previous study actually documented a significantly shorter duration for relaxation in muscles from Thoroughbreds with RER.[7] Therefore, we conclude that additional investigation of the Ca^{2+}-ATPase function as the primary site of a defect responsible for RER in Thoroughbreds is not warranted at this time.

In the study reported here, we were unable to explain the biochemical basis for the in vitro increased sensitivity to halothane-, potassium-, or caffeine-induced contracture that is characteristic of muscles from Thoroughbreds with RER or the in vivo recurrent episodes of exertional rhabdomyolysis that is characteristic of muscles of Thoroughbreds with RER. Because contractile activity of muscle is dependent on an increase in myoplasmic Ca^{2+} concentration, leading to activation of the contractile proteins on the thin filaments, the most likely explanation for an increased sensitivity to contracture for muscle fibers from horses with RER is an increased Ca^{2+} concentration in muscle from horses with RER, relative to that for muscles from control horses, in response to various agents or conditions. This still could conceivably be true, even though we have not detected an alteration in the SR RyR or Ca^{2+}-ATPase in muscle membrane preparations from horses with RER. For example, a defect in the transverse-tubule dihydropyridine receptor also could logically lead to altered RyR activity in situ and result in increased contracture sensitivity.[18] However, in vitro biochemical assays to examine the role of this protein as the voltage sensor in excitation-contraction coupling are not available. Another possible explanation for the increased contracture sensitivity of muscles from horses with RER is an increased resting myoplasmic calcium concentration relative to that for control horses. This could result from defects in other Ca^{2+}-extruding proteins in the muscle sarcolemma, such as the Na^+-Ca^{2+} exchanger and Ca^{2+}-ATPase, which also are difficult to study and have not been examined in horses with RER. Lastly, it also is possible that the physiologic data on whole muscle bundles indicating an excitation-contraction coupling or Ca^{2+}-regulation defect in Thoroughbreds with RER is not the primary defect but, rather, represents a secondary response to another alteration in muscle contractility, which cannot be replicated in isolated membrane preparations. Nevertheless, we conclude that RER in Thoroughbreds may be a novel neuromuscular disorder. The study of this condition in horses could provide information on the biochemical and genetic defects responsible for forms of MH in humans for which a genetic basis has not been identified.

[a]Sigma Chemical Co, St Louis, Mo.
[b]Nen Life Science Products, Boston, Mass.
[c]Calbiochem, La Jolla, Calif.
[d]HA membrane, Millipore, Bedford, Mass.
[e]GF/B filters, Whatman, Maidstone, UK.
[f]Model M-24, Brandel, Gaithersburg, Md.

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