Changes in Ryanodine-Induced Contractures by Stimulus Frequency in Malignant Hyperthermia Susceptible and Malignant Hyperthermia Nonsusceptible Dog Skeletal Muscle

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ABSTRACT
Elective diagnosis of malignant hyperthermia depends on halothane and caffeine contracture testing of biopsied skeletal muscle. Ryanodine-induced contractures may provide greater sensitivity and specificity for malignant hyperthermia (MH) diagnosis. This study investigated the effects of ryanodine concentration and stimulus frequency to distinguish between MH susceptible (MHS) and MH non-susceptible (MHN) dogs. Increasing ryanodine concentrations (1, 2.5 and 5 μM) increased peak isometric contracture tension, but similar responses in MHS and MHN muscle precluded use for diagnosis. Time to tension onset and to peak tension decreased with increasing ryanodine concentration, and these times were shorter in MH skeletal muscle. Increasing stimulus frequency (0.1, 0.5 and 1 Hz) decreased the time to tension onset and to peak tension, but the effect was greater in MHN muscle which decreased the difference between MHN and MHS muscle responses. When ryanodine contracture tension onset time was selected to detect MHS muscle, combinations of either 0.1 Hz and 1 μM ryanodine or 0.5 Hz and 1 μM ryanodine reduced the probability of a false diagnosis to less than 1%. Similar studies performed on human muscle might identify optimal stimulus frequency and ryanodine concentration for detecting MH in patients.

MH, a pharmacogenetic disease affecting skeletal muscle, is induced by general, volatile anesthetics and by depolarizing muscle relaxants. Before the clinical use of the skeletal muscle relaxant dantrolene sodium, the mortality from MH was > 70%. The syndrome is expressed as a hypermetabolic state with as much as 5-fold increases in oxygen consumption, causing high CO₂ production, metabolic and respiratory acidosis, high plasma CK concentration and myoglobinuria due to rhabdomyolysis. The primary cause is an abnormal sustained increase in myoplasmic [Ca<sup>2+</sup>] which produces the hypermetabolic state and in some cases, skeletal muscle rigidity (Britt and Kalow, 1970). The mechanism of MH is not completely understood but several electrophysiological, pharmacological and biochemical studies indicate abnormal release of Ca<sup>2+</sup> from the ryanodine receptor calcium release channel (Ry1) (Otsu et al., 1994, Carrier et al., 1991, Mickelson et al., 1988; Lopez et al., 1988, Nelson, 1983). Several different single amino acid changes in the protein Ry1 have linked mutation in the Ry1 gene to MH susceptibility in pigs (Fujii, et al., 1991) and humans (MacLennan et al., 1990, McCarthy et al., 1990, Gillard et al., 1991, Otsu et al., 1994). These mutations may be responsible for altering the wild-type channel into a channel which has higher Ca<sup>2+</sup> permeability and is more susceptible to physiological and pharmacological activators.

The Ry1 calcium channel is a large homotetrameric protein in which each subunit binds 1 molecule of ryanodine (Coronado et al., 1994, for review). High (nanomolar) and low (micromolar) affinity ryanodine binding sites have been described (Pessah and Zimanyi, 1991) and binding of ryanodine to these sites is very complex because of interaction with a negative cooperativity (Carroll et al., 1991; Lai et al., 1989).

Binding to the high affinity site is associated with a sustained, open substate channel conductance whereas binding to the low affinity site produces inactivation of the channel (Lai et al., 1989; Zimanyi et al., 1992).

The CHCT is the method accepted by The North American and European MH Group to detect patients susceptible to MH (Larach, 1989; The European Malignant Hyperpyrexia Group, 1984). This method evaluates critical tensions induced by caffeine and/or halothane in biopsied, human skeletal muscle. Although the CHCT continues to be important

ABBREVIATIONS: MHS, malignant hyperthermia susceptible; MHN, malignant hyperthermia nonsusceptible; Ry1, ryanodine receptor calcium release channel; CHCT, caffeine-halothane-contracture test; RCT, ryanodine-contracture test; Pt, maximal tension amplitude; CSA, cross-sectional area; MHE, malignant hyperthermia equivocal.
for the diagnosis of MH susceptible individuals, its sensitivity and specificity have been questioned; especially after discovery of several different Ry1 MH mutations (Deufel et al., 1995; MacLennan, 1995). With the aim of improving the in vitro contracture test, the European MH group suggested the use of an RCT in addition to the CHCT (Wappler et al., 1996). Data from these laboratories show that RCT is more specific for detecting MH equivocal patients; however, some overlaps between MHN and MHS patients occurred, especially at higher ryanodine concentrations (i.e., > 2 μM) (Lenzen et al., 1993). Recently, Wappler (Wappler et al., 1996) demonstrated that the overlap can be reduced at lower ryanodine concentration (1 μM).

Experiments using different methods show that the binding of ryanodine to Ry1 is very slow (Pessah and Zimanyi, 1991). This time can be reduced by pre-activation of Ry1 with caffeine, ATP or Ca2+ and prolonged by Mg2+ (Chu et al., 1999; Rousseau et al., 1987). Increasing the open state probability of the Ca2+ channel increases accessibility of Ry to the binding site. In relation to MH diagnostic testing, the CHCT, involving two tests, takes less than 30 minutes while the RCT takes 2 to 3 hr if 1 μM of Ry is used. In our study we tested a hypothesis that ryanodine binding is use dependent and that by increasing the frequency of muscle stimulation the time to ryanodine-induced contracture tension would be shortened. If correct, this method could be used to reduce the time of RCT for MH diagnosis and possibly improve diagnostic sensitivity and specificity. The objective of our study was to investigate if the kinetics of ryanodine-induced contracture in intact muscle fibers could be changed by use-dependence mechanisms and, if so, then to determine the stimulus frequency and ryanodine concentration optimal for distinguishing MHN from MHS.

**Methods**

**MH phenotyping.** This study was performed in nine nonsusceptible (MHN) and seven MHS dogs. The experimental protocol was approved by our Institutional Animal Care and Use Committee. MH susceptibility of each dog was determined by an in vivo test with a halothane-succinylcholine challenge protocol (Nelson, 1991) and by the in vitro CHCT (Larach, 1989). During the in vivo test, body temperature, muscle rigidity, end tidal CO2 level and arterial blood gases were evaluated. For in vitro testing, specimens from gracilis muscle were biopsied, under pentobarbital anesthesia, on the same day and immediately before the in vivo test and submitted to the contracture protocol approved by the North American MH Group for human MH diagnosis (Larach, 1989).

**Preparation of muscle specimens for ryanodine study.** At least 1 mo after the MH phenotyping, each dog was anesthetized with pentobarbital, 25 mg/kg, i.v., the trachea intubated and the lungs mechanically ventilated with 70% O2 and 30% N2O. During this anesthesia, 14 to 16 muscle specimens (length = 2–3 cm, width = 1.5–3 mm, weight = 0.1–0.2 g) were dissected from the gracilis muscle of each animal. Four specimens were mounted in 50 ml vertical chambers, filled with Krebs-Ringer solution (in mmol/liter, NaCl, 118.1; KCl, 3.4; MgSO4, 6.8; KH2PO4, 1.2; glucose, 11.1; NaHCO3, 25; CaCl2, H2O, 2.5, pH 7.4), saturated with carbogen (95% O2/5% CO2) and the temperature maintained at 37°C. Others specimens for later testing were stored in Krebs-Ringer solution at room temperature and continuously oxygenated until used. One end of each fascicle was tied to a fixed clamp and the other end attached to a FT-03 force transducer (Grass Instruments, West Warwick, RI) mounted on a micromanipulator. The muscles were electrically stimulated by a pair of platinum electrodes connected to a Grass model 888 stimulator. To maximize twitch tension each fiber received a different current via a Med-Lab Stimu-Splitter II connected between the Grass stimulator and platinum electrodes. The muscle length was adjusted to obtain twitches of Pt. The frequency and duration of stimulation were 0.1 Hz and 2 msec, respectively, during the period of muscle preparation. The total time of each experiment varied from 3 to 7 hr. Over the period of these experiments, the muscle twitch was not significantly affected. The muscle twitches were recorded by a Grass model 7400 (Astro-Med, Inc, Instr, West Warwick, RI) instrument. The analog signal was conditioned and digitized by a Cyber-Amp 320 Programmable Signal Conditioner and Digidata 1200 Interface, respectively (Axon Instruments, Foster City, CA). The data were further analyzed using pClamp6 software (Axon Instruments).

**Experimental protocol.** To investigate the effect of use-dependence on the kinetics and intensity of ryanodine-induced contractures, 12 viable fascicles were divided in 3 sets of 4 fascicles each. Each set was stimulated at a fixed frequency (0.1, 0.5 or 1 Hz) and each fascicle exposed to a fixed ryanodine concentration (0, 1, 2.5 or 5 μM). The ryanodine concentration in the bath was randomly selected. Therefore, each specimen was stimulated at a fixed frequency and tested with a fixed concentration of Ry. The control (0 ryanodine) was necessary to measure the effect of time and stimulus frequency on the baseline tension of the muscle specimen. After adding ryanodine into the Krebs-Ringer solution, we determined the time to tension onset, half-time to peak tension and time to peak tension. We also correlated the tension with the time of exposure to Ry. The peak tension was also correlated to Pt measured just before adding Ry into solution and to CSA calculated at the end of experiments using the equation: CSA (cm2) = weight (g)/length (cm) × 1.06 g/cm2.

The fascicles were exposed to Ry during the time necessary to reach the contracture plateau. Then, the ryanodine solution was replaced by a fresh Krebs-Ringer solution without ryanodine. We did not wait to complete recovery of tension to baseline, except for a few experiments where we desired to investigate the offset time of ryanodine from the Ry1.

**Drugs.** High purity ryanodine was purchased from Calbiochem and the stock solution was prepared by dissolving the powder in distilled water and frozen until used.

**Data collections and analysis.** The time to onset tension was determined at the moment that the trace starts to rise from the baseline after addition of ryanodine into solution. The values are presented as mean ± S.E. The data from MHN and MHS dogs were compared between groups by Student’s t test.

**Results**

**Effect of ryanodine concentration.** Exposure of normal and MHS dog gracilis muscle to increasing concentrations of ryanodine produced slowly developing isometric contractures that increased with increasing ryanodine concentration (fig. 1). In normal dog muscle 1 μM ryanodine produced an average peak contracture tension of 2.07 ± 0.49 g, and the tensions at 5 μM ryanodine averaged 4.11 ± 0.77 g. The average tensions produced by 1, 2.5, and 5 μM ryanodine in MHS dog muscle were greater than those in normal muscle but these were not statistically significantly different (fig. 1). The time to onset of ryanodine contracture tension was inversely affected by increasing ryanodine concentrations (fig. 2). In MHN muscle the time to 1 μM ryanodine-induced tension onset was almost 1 hr although at 5 μM the time to tension onset was reduced to 19 min. This effect of ryanodine concentration on time to tension onset was much greater in MHN compared to MHS muscle (fig. 2). The tension onset time was 10 times shorter for 1 μM ryanodine in MHS muscle; i.e., 5.5 ± 0.9 min in MHS vs. 55 ± 10.4 min for MHN.
Because of the shorter time to tension onset in MHS muscle, increasing the concentration of ryanodine had less effect in this muscle. The difference in time to tension onset between MHS and MHN muscle is greater at 1 μM ryanodine and diminishes as the concentration of ryanodine is increased (fig. 2). Similar to the time to onset of ryanodine contractures, the time to peak contracture was inversely affected by ryanodine concentration and was shorter for MHS muscle (fig. 3). At 1 μM ryanodine the average time to peak tension was 152.7 ± 11.7 min for MHN and 36.4 ± 8.1 min for the MHS muscles. Increasing the ryanodine concentration from 1 to 5 μM decreased the time to peak tension by approximately 50% in both MHN and MHS muscle (fig. 3).

**Effect of stimulus frequency on ryanodine contractures.** Increasing the frequency of electrical stimulation dramatically decreased the time to onset of ryanodine-induced contracture in MHN but had little effect in the MHS dog muscles (fig. 4). This effect of increasing stimulus frequency was greater at the lower concentrations of ryanodine where increasing from 0.1 to 1 Hz caused decreases of 74, 71 and 68% in the time to tension onset at 1, 2.5 and 5 μM ryanodine respectively. In comparison, this same increase in frequency produced decreases in time to tension onset of only 10.8, 22.2 and 3% at 1, 2.5 and 5 μM ryanodine-induced contractures of MHS muscle. The time to peak tension was also decreased by increasing the rate of stimulation (fig. 5). In MHN muscles, a 10-fold increase in stimulus frequency decreased the time to peak contracture by 65, 65.3 and 60.9% at 1, 2.5 and 5 μM ryanodine. A similar, but smaller effect of increasing stimulus frequency was observed in the MHS muscle where a 10-fold increase in frequency produced 52, 45.7 and 42.4% decreases in the time to peak tension at 1, 2.5, and 5 μM ryanodine. The peak tension produced at each ryanodine concentration was not significantly affected by the rate of stimulation of either MHN or MHS muscle (fig. 5). Correcting the tension for grams per cross-sectional area or for Pt of each fascicle did not alter these responses (data not shown).
Caffeine and halothane cause Ca\(^{++}\) release from the SR membrane by activating the Ry1 calcium release channel. Exposure of biopsied skeletal muscle to these compounds and measurement of the critical contracture tension provides the basis for the North American and European MH diagnostic centers' identification of MH susceptible patients (Larach, 1989; The European Malignant Hyperpyrexia Group, 1984). A third drug, ryanodine, has been introduced by the European MH group to improve the separation between MHN, MHE and MHS responses (Wappler et al., 1996). Ryanodine binds specifically to the Ry1 calcium release channel and probably causes contracture in skeletal muscle by induction of a sustained open, subconducting state of the Ry1 channel, allowing Ca\(^{++}\) to slowly leak from the SR (Rousseau et al., 1987). Because mutations in Ry1 link to MH in some families, it might be expected that ryanodine-induced contractures might provide greater sensitivity and specificity for MH diagnostic contracture testing. Preliminary results from the European group show that the RCT is effective for distinguishing MHN from MHS patients and for reducing, but not abolishing the number of equivocal tests detected by the CHCT (Hopkins et al., 1991; Wappler et al., 1996). In contrast, others studies showed that improvement of the RCT protocol would be necessary to reduce overlap between MHN and MHS responses (Wappler et al., 1996, Hartung et al., 1996). In our study we demonstrate that the amplitude and kinetics of ryanodine-induced contracture can be changed by altering ryanodine concentration and/or frequency of electrical stimulation in MHN but not in MHS specimens.

**Ry Contracture: MHN vs. MHN Dogs**

**Ry concentration vs. tension.** Using the MH dog model we found that measurement of peak tension in response to 1, 2.5 and 5 \(\mu M\) ryanodine was not a reliable method for detecting MHS in the dog. There was no significant difference between the two groups across the range of Ry concentrations tested (fig. 1). We considered the possibility that variability of the data could be related to specimen size or viability. However, correction of the peak tension values for CSA or for the Pt did not improve the difference between MHN and MHS responses. We conclude that the ryanodine-induced peak tension values obtained in this study are not useful for separating MHN from MHS dog muscle. We observed a better and more reliable differentiation when time variables, instead of absolute tension, were considered. At 1 \(\mu M\) Ry the time to onset of contracture tension in MHS dog muscle was 10 times shorter than for MHN muscle, and at 1 and 2.5 \(\mu M\) ryanodine no overlap occurred between the groups (fig. 2). The time to peak tension in response to all tested ryanodine concentrations was also a very reliable indicator (fig. 3). However, measuring the time to ryanodine-induced peak tension for MH diagnostic purposes may add several hours to the total testing time. This could have adverse effects on muscle viability when the additional time required for the CHCT is also considered. Thus, using time to peak ryanodine-induced tension as an adjunct diagnostic test may not be advantageous.

**Stimulation frequency versus Ry contracture tension and kinetics.** The use-dependence for Ry-induced contracture was tested by changing the frequency of stimulation. Significant decreases in the tension onset time and time to peak tension were observed when the frequency was increased from 0.1 to 0.5 Hz. However, the higher the frequency, the worse the separation between MHN and MHS specimens.
dog muscle responses. This is primarily due to MHS muscle responses not being affected by stimulus frequency and to the movement of MHN response values closer to the Ry response values. The advantage of using higher frequency for MH diagnosis is the reduced time required to complete the test, but if too high a frequency is used, the power of diagnostic discrimination is reduced. If onset time was chosen as the variable to detect MH patients, the probability of having a false negative with the combinations of 0.1 Hz and 1 μM Ry or with 0.5 Hz and 1 μM Ry was lower than 1%. In the MHS group the onset time for all fascicles used in this study was less than 10 minutes with very small variability (fig. 4). Therefore, if we select 10 min as the time to contracture tension onset for a positive MH diagnosis, the probability of obtaining a false negative would be < 1%. This time of muscle exposure to Ry is close to the exposure time for the halothane test and is shorter than that for the caffeine test in the CHCT.

The frequency-dependence for ryanodine contractures in human muscle should be investigated because it may be different from that in dog muscle. Studies from the European group have only changed the Ry concentration (Wappler et al., 1996; Hartung et al., 1996) and have fixed the frequency at 0.2 Hz. It could be that in human muscle the overlap between MHN and MHE could be decreased by lowering frequency of stimulation from 0.2 to 0.1 Hz.

### Variability in Ry responses.

Regarding sources of variability in the data, it is interesting to note that variability was smaller for time to peak tension than it was for time to tension onset. Variability was also smaller in MHS than MHN responses; a discrepancy that has also been observed in human muscle (Hartung et al., 1996). Hartung et al. (1996) described higher variability in muscle fascicle responses among control and MHN groups than in MHS fascicles. They also found the smallest variability in the MH fulminant group of patients clinically classified as grade 6 with raw score range ≥ 50 (Larach et al., 1994). The exact onset time is usually more difficult to determine than is time to peak tension because the development of Ry-induced tension is very slow. Consequently it is difficult to determine exactly when the trace starts to rise from the baseline because there is not a clear deflection. Another explanation for variability could be related to different sensitivities of the normal Ry1 population. This sensitivity could be modulated by activation level or by some conformational change of the Ry1 caused by mutations other than those predisposing to MH.

### Mechanisms of Ry-induced tension and factors affecting them.

Different mechanisms could be involved to explain the acceleration of Ry-induced contracture caused by Ry concentration and by frequency of stimulation. Binding analysis using [3H]Ry has shown low association and dissociation rates that require long incubation periods for analysis (Hawkes et al., 1992). Electrophysiological studies measuring Ry activity of incorporated channels in a lipid bilayer show that Ry increases the open state probability and maintains the Ca++ channel in sustained open substate (Lai et al., 1989; Zimanyi et al., 1992). In intact muscle exposed to low concentrations of Ry, the slow binding of Ry and partial activation of the channels results in a slow rate of Ca++ release into the sarcoplasm allowing most of it to be pumped back into the SR by Ca-ATPase. The dissociation of Ry is also slow, and over time there is a cumulative effect as more Ry1 molecules are bound. As more and more of the four Ry1 binding sites become occupied per molecule, more Ca++ is released, requiring more Ca++-ATPase activation. With time, the rate of Ca++ release exceeds that for Ca++ uptake, resulting in a critical level of Ca++ that activates the contractile proteins and leads to tension onset.

The rate of Ry-induced tension was significantly increased by increasing the frequency of stimulation. In our interpretation, Ry binds to the receptor when the Ca++ channel is activated to an open state. For this reason, tension is developed slower at low stimulus frequency or in the absence of stimulation. Several others studies have demonstrated that the affinity of Ry1 can be changed by experimental conditions. Binding of [3H]Ry and Ca++ channel activation is optimal at 10 to 100 μM Ca++ concentration and inhibited in mM range (Chu et al., 1990). Preactivation of Ry by caffeine increased the rate of [3H]Ry association without changing the rate of dissociation (Chu et al., 1990; Zimanyi et al., 1992). In skinned skeletal and cardiac fibers, Su (1992a; 1992b) reported that the combination of caffeine with Ry decreased the Ca++ sequestration in the SR. This effect was caffeine dose dependent.

In MHS specimens the onset time and the time to peak were not affected by either Ry concentration or by frequency of stimulation. Assuming that the greater sensitivity of MHS dog muscle to ryanodine-induced contracture is a consequence of higher affinity for Ry binding and number of binding sites, we can question why neither Ry concentration nor stimulus frequency affected the onset time of MHS muscle. We conclude that the Ry1 channels in MHS muscle are in an optimal configuration for binding even when stimulus frequency is low. The most likely explanation for this is that the MHS mutation favors the binding of Ry. Therefore, increasing frequency of stimulation minimizes the separation of MHN from MHS dog muscle. In human muscle it may be that decreasing stimulus frequency from 0.2 Hz to 0.1 Hz or less will improve the ability to distinguish between MHN from MHS muscle. Further studies comparing ryanodine contractures in MHS and MHN human skeletal muscle will be necessary in order to determine the optimal concentration, stimulus frequency, and so on for the use of ryanodine in the diagnosis of MH in humans.

### References


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