Malignant Hyperthermia: A Pharmacogenetic Disease of Ca++ Regulating Proteins

Thomas E. Nelson*

Department of Anesthesiology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, North Carolina 27157-1009, USA

Abstract: Malignant hyperthermia (MH) is a pharmacogenetic, life-threatening hypermetabolic syndrome in genetically predisposed individuals exposed to certain anesthetic agents. Discovered by Denborough and Lovell [1] in 1960, MH was associated with high mortality and morbidity as the cause was unknown and an effective treatment was unavailable. There is no classic clinical presentation of the syndrome, and the onset and signs of MH are dependent upon known and unknown environmental and genetic factors. Initial theories involved central temperature regulation defects or uncoupling of oxidative phosphorylation in mitochondria [2], but later investigations targeted skeletal muscle as the affected organ. Subsequently freshly biopsied skeletal muscle was used for in vitro pharmacologic contracture testing to discriminate between normal and MH-affected muscle and remains the “gold standard” for MH diagnosis. Spontaneous, genetic models for MH were discovered in pigs and dogs and substantial knowledge about MH was gained from these valuable resources. The abnormal contracture response of MH skeletal muscle evoked a focus on calcium regulation, and abnormalities in calcium release (as opposed to calcium sequestration) mechanisms were discovered. About this same time the major calcium release channel in the skeletal muscle sarcoplasmic reticulum membrane was purified and named the ryanodine receptor [3]. Although the ryanodine receptor represents one of the largest functional proteins, the enormous gene encoding the 5021 amino acids comprising the ryanodine receptor subunit was eventually cloned [4,5]. Patient and dedicated work on the ryanodine receptor gene has found linkage to MH in the pig [6], dog [7], and among several different mutations and MH in unrelated human families [8,9]. Expression of these mutations in HEK cells has resulted in abnormal calcium release [10,11], supporting but not proving a causal basis for MH. In this review each of the areas mentioned above is discussed in detail revealing a wonderful success story that changed the anesthesiologist’s “worst nightmare” from a syndrome with high mortality and morbidity to a reasonably well managed disease today. This success story includes unraveling the molecular basis for the disease and brings its pathoetiologic and diagnostic aspects toward molecular genetic resolution.

Key words: dog, genetics, human, hyperthermia, pig, review, ryanodine, RyR1

DISCOVERY

Some credit for discovery of malignant hyperthermia (MH) in 1960 should go to a patient who communicated appropriately by calling his physicians’ attention to the fact that 10 of his close relatives had died unexpectedly during general anesthesia. Consequently, this patient was reluctant to undergo general anesthesia for repair of a compound fracture, and he was able to draw attention to his concerns. In spite of reassurances, the patient did develop the MH hypermetabolic syndrome, was packed in ice, treated symptomatically, and survived. Medical records revealed that his relatives had experienced similar episodes, which prompted Denborough and Lovell [1] to publish a report of the case and to suggest that the syndrome was familial with possible dominant inheritance. Prior to this report there was documentation of “ether convulsions” associated with excessive rises in temperature, suggesting that MH was occurring but its familial aspect went unrecognized [12,13]. In the late 1960s two investigators at the University of Toronto, Drs. Werner Kalow and Beverly Britt, teamed up for research on MH, and their contributions to the field are notable. Initially they investigated a large, 450-member Wausau, Wisconsin, family in which 20 episodes of MH with 8 fatalities were found. The data from this family also suggested a pattern of autosomal dominant inheritance [14]. From this time forward, many case reports of MH episodes began appearing in the literature, leading to universal acceptance of MH as an inherited trait predisposing...
to the anesthetic agent-induced hypermetabolic syndrome. Another significant contribution by Kalow and Britt to MH discovery was the finding that freshly biopsied skeletal muscle from MH susceptible survivors had abnormal contracture responses to caffeine, a response that was potentiated by halothane [15]. The significance of this important discovery will be discussed in more detail under etiology and diagnosis. Another historically critical discovery in MH was the finding by Ellis et al. [16] that halothane alone could cause abnormal contracture in MH skeletal muscle. These abnormal muscle contracture responses provided the background for considering a peripheral rather than a central origin for MH and later would become the basis for elective diagnosis of MH. Even after MH was acknowledged as an inherited trait, patients continued to die from this disease (mortality ~70%) because it always occurs unexpectedly and there was no effective treatment. A landmark discovery by Harrison [17] determined that the unique, direct-acting skeletal muscle relaxant dantrolene was efficacious in treating MH. Soon afterwards the availability of an intravenous form of this drug [18] proved to be a remarkable life-saving event in the history of MH. The next major discoveries involved a) pinpointing the cause of MH, which involved dysregulation of calcium in MH skeletal muscle, b) identifying the intracellular calcium release channel as the defective site, and c) cloning the RYR1 calcium channel gene with subsequent discoveries of mutations in this gene linking to MH in humans, pigs, and dogs. The ultimate and final discoveries for MH will be identification of all mutations causing the disease and a simple, noninvasive test to identify the MH susceptible population. In the following sections details about how the events listed above unfolded will be provided.

**CLINICAL MH SYNDROME**

There is no classic clinical presentation of the MH syndrome and what occurs is dependent on known and unknown genetic and environmental variables. The normal physiologic response to general anesthesia is decrease in heart rate, decrease in metabolic rate, and decrease in body temperature. Administration of depolarizing muscle relaxants such as succinylcholine produces muscle paralysis. When an apparently normal, healthy individual responds to general anesthesia by inappropriate tachycardia, a 5-fold increase in oxygen consumption, profound metabolic and respiratory acidosis, and perhaps skeletal muscle rigidity, the anesthesiologist is confronted with anesthetic-induced MH. However, not all of these signs will occur and the rate at which they develop can vary considerably. Development of the MH syndrome is dependent upon the anesthetic agents used, the patient’s core temperature and genetic predisposition, as well as other unknown epigenetic forces. The anesthetic drugs that trigger MH and those which are safe have been established (Table 1), so that if an individual is known to be malignant hyperthermia susceptible (MHS), safe drugs can be selected to provide adequate anesthesia.

**Table 1. Malignant hyperthermia - trigger and safe anesthetic agents.**

<table>
<thead>
<tr>
<th>Trigger Agents</th>
<th>Safe Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhaled Anesthetics</strong></td>
<td><strong>Inhaled Anesthetics</strong></td>
</tr>
<tr>
<td>Halothane</td>
<td>NO2</td>
</tr>
<tr>
<td>Isoflurane</td>
<td>Xenon</td>
</tr>
<tr>
<td>Enflurane</td>
<td>Propofol</td>
</tr>
<tr>
<td>Sevoflurane</td>
<td>Ketamine</td>
</tr>
<tr>
<td><strong>Intravenous Anesthetics</strong></td>
<td><strong>Barbiturates</strong></td>
</tr>
<tr>
<td>None</td>
<td><strong>Local Anesthetics</strong></td>
</tr>
<tr>
<td></td>
<td>All</td>
</tr>
<tr>
<td><strong>Muscle Relaxants</strong></td>
<td><strong>Muscle Relaxants</strong></td>
</tr>
<tr>
<td>Depolarizing</td>
<td>Nondepolarizing (All)</td>
</tr>
<tr>
<td>(succinylcholine)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Benzodiazepines</td>
</tr>
</tbody>
</table>

The most intense trigger for MH is considered to be induction of anesthesia with a potent volatile agent followed by intravenous succinylcholine. The combination of these agents can produce an immediate onset of the MH syndrome with global skeletal muscle rigidity, tachyarrhythmias, and rapid increases in body temperature and oxygen consumption. In contrast, if in that same MH susceptible individual anesthesia is induced by intravenous administration of a barbiturate followed by succinylcholine for muscle paralysis, a completely normal response may occur until the potent volatile agent is introduced to maintain anesthesia. After this the MH syndrome may develop either rapidly, slowly over an hour or longer, or it may not develop at all. Many MH susceptible individuals have received multiple “MH-trigger” anesthetics without developing MH but upon receiving subsequent anesthesia (as many as 10 different exposures) the MH syndrome develops. In general, the earliest signs of MH are increases in expired carbon dioxide and heart rate which are associated with increased metabolism. If the triggering agents continue to be administered, the rate of metabolism increases exponentially with the consequences being profound metabolic and respiratory acidosis, rapid rises in body temperature, and electrolyte disturbances. The initial hypermetabolic state originates in skeletal muscle, and consequent rhabdomyolysis produces hyperkalemia and myoglobinuria. If myoplasmic
calcium rises substantially during the MH crisis, global skeletal muscle rigidity will occur. The acidosis and electrolyte disturbances ultimately lead to lethal cardiac arrhythmias. Reduced mortality and morbidity from MH depend upon prompt diagnosis, discontinuation of triggering anesthetics, and treatment. An example of clinical MH in a young patient is illustrated in (Figure 1).

CENTRAL CORE DISEASE AND MH: SINGLE OR MULTIPLE IDENTITIES?

Central core disease (CCD) is a non-progressive myopathy which may have variation in clinical presentation. CCD is inherited as an autosomal dominant trait with variable penetrance and expressivity. The predominant clinical features are hypotonia and delayed motor milestones in infancy with mild proximal weakness affecting mainly the lower limbs. The designation CCD derives from the histochemical presence of a central zone (core) in muscle fibers which is devoid of oxidative and phosphorylase enzyme activity and contains virtually no mitochondria [19]. The presence of central cores is limited to type I myofibers. The occurrence of MH in individuals with CCD [20,21] has encouraged the treatment of all individuals with CCD as being susceptible for MH.

The significant question is whether MH and CCD represent variations in phenotypes among different mutations within a single gene or if variations in genotypes exist. The genetic similarity between MH and CCD is that they share allelic identity by having a mutation in RYR1 but they are phenotypically different by the presence of cores in the type I myofibers of CCD individuals. The cause for the phenotypic differences between MH and CCD individuals remains unresolved but may be due to polygenic causes (mutations in addition to the recognized RYR1 mutation) or it may be that the CCD mutations cause a more severe disruption in myoplasmic [Ca++] regulation that produces the different phenotype. In CCD it has been postulated that the central cores are formed because the center of the fiber has fewer calcium regulating proteins compared to the periphery [22]. Thus, in CCD the abnormal increased [Ca++] in the central core forces mitochondria to remove the extra calcium and this leads to calcium overload and destruction of the mitochondria and surrounding proteins. If MH and CCD are both caused by a dysregulation of myoplasmic [Ca++] which links to an single mutation in RYR1, then perhaps they should all be designated as MH with varying phenotypic expression. Presently, MH linked to mutations in RYR1 and in the DHPR gene are collectively referred to as MH and therefore it is not clear why CCD and

---

**Figure 1.** An example of fulminating malignant hyperthermia (MH) in a 15-year-old patient and its successful treatment. Anesthesia was induced with propofol, muscle paralysis by mivacurium and anesthesia maintained with isoflurane. Upon diagnosis of MH, isoflurane was discontinued and treatment with dantrolene, bicarbonate and ice packs produced a rapid reversal of the syndrome. The patient survived without sequelae.
MH are considered as separate entities. As the complete genetic basis for MH is resolved in the future, appropriate genetic guidelines will resolve these issues.

TREATMENT OF THE MH SYNDROME

By the early 1970s many anesthesia providers were aware of MH, and when it occurred an early diagnosis and symptomatic treatment was associated with reduced morbidity and mortality. However, if the hypermetabolic syndrome had progressed to a significant degree before the diagnosis was made and only then was treatment initiated, the mortality rate was higher [23]. Without an effective treatment, mortality and morbidity remained significant. Although most early treatments of MH were symptomatic, therapies aimed at the hypermetabolic aspects of the syndrome were attempted with very little success [24]. As data supporting the theories of abnormal increased skeletal muscle cell calcium as the primary event for MH developed, therapies were directed at controlling calcium. Calcium antagonists used in an attempt to block calcium release included local anesthetics [25-27] and magnesium [28,29]. For a brief period, procaine or procainamide was recommended for treatment of MH, but studies in MH pigs showed these to be either partially effective or ineffective [30,31]. Intravenous magnesium attenuated but did not prevent MH in pigs [29].

The report by Harrison [32] that the skeletal muscle relaxant dantrolene was used effectively in pigs led to one of the most significant advances in MH; i.e., discovery of a highly efficacious drug for treatment or prevention of MH. In search of centrally acting drugs to treat spasticity, Snyder et al. [33] synthesized 1-[(5-Arylfurfurylindene)amino] hydantoins and measured their ability to block flexor reflexes in the hind limb of anesthetized cats. In the study they discovered some inhibition of the response to motor nerve stimulation. Further studies [34,35] demonstrated that dantrolene 1-[(5-(4-nitrophenyl)-2 furyl)] methylene] amino] -2,4-imidazolidinedione produced its skeletal muscle relaxant effects by blocking calcium release from inside the muscle cell, indicating a unique mode of action. Subsequent investigations [31,36-38] confirmed the efficacy of dantrolene for prophylaxis and therapeutics of MH in pigs, setting the stage for its use in humans. An intravenous form of dantrolene was not available, and in desperation oral capsules were opened and dissolved when MH cases appeared. An intravenous formulation was manufactured and approved for human use by the FDA, but the dose remained empirical until animal [38] and human [39] studies determined that the ED₉₅ dantrolene dose for muscle twitch depression was prophylactic and therapeutic for MH. An interesting action of dantrolene is that it cannot completely block electrically-evoked contractions and produces a maximum block of approximately 70% [35]. The basis for this incomplete block of skeletal muscle contraction remains unexplained, but it is sufficient to prevent the anesthetic drugs from releasing calcium inside the MH muscle cell.

When MH is diagnosed, an initial dose of 2.5 mg/kg is administered, and if symptoms do not abate, up to 10 mg/kg can be administered. Other treatments to control MH-associated acidosis, hyperkalemia, hyperthermia, and myoglobinuria are well described in standard anesthesiology textbooks and on the MHAUS website (www.MHAUS.org). Current recommendations are that each surgical facility contain sufficient supplies of dantrolene for the immediate treatment of MH when it occurs. As a consequence of education about MH and the availability of dantrolene, the mortality from MH has been reduced from 70% to <1%. The evidence that dantrolene acts directly on the RyR1 calcium release channel to block calcium release will be discussed in detail in the section on the ryanodine receptor.

Animal Models for MH

Pigs

As with other human diseases, the advancements in reducing mortality and morbidity from MH have been markedly facilitated by genetic animal models. Major contributions have been made through investigations in malignant hyperthermia susceptible (MHS) pigs. Intense genetic selection for increased muscle mass in pigs for improved pork production resulted in selection for a gene that predisposed the animals to stress syndromes. This was first recognized in the pork industry when after slaughter some meat had the appearance of pale, soft, exudative pork (PSEP). One of the earliest descriptions of this was by Ludvigsen in 1954 [40]. In addition to post-slaughter changes in meat quality, some of these animals died from a stress-induced syndrome which was triggered by a variety of stressors ranging from breeding, fighting to establish pecking order, to shipping stress [41]. Some breeds of swine had a high incidence of these stress syndromes which resulted in considerable economic losses. The first case of anesthetic agent-induced MH in pigs was reported by Hall et al. [42]. Eventually an association between the stress syndromes and anesthetic-induced MH was suspected [43,44]. These suspicions were eventually proven by various experiments [45,46]. A systematic investigation comparing the anesthetic-induced clinical syndrome in pigs to that reported in man demonstrated that the MHS pigs could serve as a useful animal model [47]. The MHS pig model contributed to new knowledge about MH in many respects, including etiology, treatment, and inheritance. Early experiments focused on the biochemistry of heat production during MH in pigs with considerable debate regarding aerobic versus
anaerobic heat production. Subsequently, pharmacologic investigations of MH pig skeletal muscle revealed that abnormal \textit{in vitro} contracture sensitivity to caffeine and halothane was similar to that reported in humans [48].

Standardized \textit{in vivo} anesthetic challenge and \textit{in vitro} diagnostic protocols [48] were sufficient to discriminate among homozygous normal and MH and MH heterozygous. The \textit{in vivo} responses to halothane clearly distinguished the MH homozygous pigs from normals and heterozygous, but multiple doses of succinylcholine plus halothane were necessary to distinguish MH heterozygous from normal pigs (Figure 2). Others [49] have reported no differences in response to anesthesia in MH heterozygotes and normals, but details regarding standardized protocols and the use of mixed breed pigs may have influenced the outcome in these studies.

**Dogs**

The higher frequency of occurrence of MH in pigs can be explained by controlled breeding practices, but in dogs there are only a few clinical cases of MH reported [50]. The colony of MH mixed breed dogs we maintained for several years originated from Canada and were started from a clinically discovered MH founder. It is not clear if these animals were related to those reported by O'Brien [51], but our results varied significantly from those reported by this group. First of all, we never experienced stress-induced MH in these animals even though they were very excitable and active. They were susceptible to all MH-trigger agents, including methoxyflurane, and they did not develop metabolic acidosis or skeletal muscle rigidity with the anesthetic-induced syndrome. A non-rigid variant of MH in humans was debated at one time, and this canine MH model shows that the MH syndrome can occur without skeletal muscle rigidity. MH is inherited as an autosomal dominant trait in humans and dogs [14,50] and as an autosomal recessive trait in pigs [47]. Expression of the fulminant MH syndrome occurs in MH homozygous but not in heterozygous pigs, indicating that the gene penetrance is stronger in MH humans than in pigs. The linkage of MHS with a mutation in RYR1 (see Genetics Section) in this dog colony is interesting from the standpoint of how a specific mutation may relate to the MH phenotype. Consequently, the MH dog may mimic MH in humans better than the MH pig. At the present time all MH susceptible species have several things in common and these are compared in (Table 2).

![Image](image.png)

**Figure 2.** Comparison of the response to halothane ± succinylcholine (SCh) administration to MH positive homozygous (nn) and heterozygous (nN) and MH negative (NN) pigs. Anesthesia was induced with barbiturate and halothane administration begun 45 min later. Halothane alone triggers MH in pigs within a 1-hour exposure as illustrated by the rapid rise in arterial blood lactate levels. No significant rise in lactate occurs in the MH heterozygous pigs until succinylcholine is administered 2 hours after starting halothane. The anesthetic challenge had no effect on blood lactate in non-susceptible pigs.
Table 2. Comparison of MH characteristics among different MHS species.

<table>
<thead>
<tr>
<th>MH Characteristics</th>
<th>Human</th>
<th>Dog</th>
<th>Pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypermetabolic syndrome with ↑Vo2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Metabolic acidosis</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Muscle rigidity +/-</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Dantrolene efficacy</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Abnormal in vitro contracture</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Halothane</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Caffeine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stress-induced MH</td>
<td>?</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Genetic inheritance</td>
<td>autosomal dominant</td>
<td>autosomal dominant</td>
<td>autosomal recessive</td>
</tr>
</tbody>
</table>

GENETICALLY MODIFIED ANIMAL MODELS

No genetic, small animal models for MH have been established. The RyR1 knockout mouse is lethal at birth due to insufficient muscle function for respiration [52], preventing animal studies. Attempts to produce MH RyR1 mutant transgenic mice have thus far failed (Dr. Paul Allen, personal communication) but these efforts are continuing. It would be of considerable value to determine if a transgenic mouse carrying one of the human MH RYR1 mutations is capable of developing the clinical MH phenotype or not.

If, for example, such a transgenic mouse develops clinical MH in response to MH-trigger anesthetics, this would indicate that other genes are not directly involved with the genetic predisposition. If, on the other hand, the MH transgenic animal does not develop clinical MH, then other genes must be important to the expression of the phenotype.

MH ETIOLOGY: MYOPLASMIC CA++ DYSREGULATION

Independent studies show that the primary event leading to the MH syndrome is an abnormal, sustained increase in myoplasmic calcium concentration ([Ca++] in affected skeletal muscle. The abnormal in vitro halothane contractures of MH skeletal muscle are prevented by incubating the muscle in the absence of calcium [53-55], and when calcium is replenished the halothane contractures appear. Dantrolene sodium, a unique, direct-acting muscle relaxant [35], is the only drug with efficacy for treating or preventing MH. In vitro, dantrolene blocks the halothane contractures [31,56] and in vivo, pretreatment of pigs with an ED_{95} twitch blocking dose prevents anesthetic-induced MH [38]. A direct measurement of myoplasmic [Ca++] during MH in pigs showed that the anesthetic-induced increase in [Ca++] preceded all early clinical signs of MH, and dantrolene produced an immediate decrease in myoplasmic [Ca++] [57]. It is now established that dantrolene binds to and blocks the RyR1 calcium release channel [58,59].

These effects of calcium and dantrolene on the in vitro and in vivo responses of MH muscle to halothane support the popular hypothesis that anesthetics cause MH by producing sustained, abnormal calcium release from the sarcoplasmic reticulum membrane. How does the anesthetic-induced increase in myoplasmic [Ca++] lead to the catastrophic MH syndrome?

NORMAL SKELETAL MUSCLE E-C COUPLING AND HEAT PRODUCTION

To understand the metabolic consequences of calcium dysregulation in MH muscle it is helpful to understand what happens during a contraction/relaxation cycle in normal skeletal muscle. The excitation-contraction coupling (ECC) mechanism begins with the muscle action potential generated by release of acetylcholine at the myoneural junction. The muscle action potential is propagated (via Na and K channel events) along the sarcolemmal membrane until it reaches the point where the sarcolemma invaginates into the interior of the muscle fiber and forms the transverse tubule. Located on the transverse tubule membranes are dihydropyridine-sensitive voltage-gated calcium channels (DHPR) that are opened by the action potential propagating through the sarcolemma into the cytoplasm of the muscle fiber.
the myoplasm when the channels open. The amount of calcium passing through the DHPRs and the rate of diffusion once inside the muscle cell is insufficient to account for the calcium transient associated with contraction. Consequently, the majority of calcium for contraction must be coming from the internal sarcoplasmic reticulum stores. The sarcoplasmic reticulum membrane exists throughout the skeletal muscle cell, but different areas of the membrane have predominant functions for calcium regulation. Juxtaposed to the transverse tubule membranes are large, bulbous sacs of the sarcoplasmic reticulum and these are named the terminal cisternae. The terminal cisternae contain the 14 kD protein calsequestrin which is a high affinity calcium binding protein that serves to sequester calcium within the terminal cisternae (Figure 3). Consequently, the majority of calcium inside resting muscle cells is contained within the terminal cisternae, and this is the major pool of calcium that is released for activation of the contractile proteins.

The terminal cisternae also contains the major calcium release channel for ECC; namely, the ryanodine receptor calcium release channel RyR1 (Figure 3). (In this review RyR1 will refer to the protein and RYR1 refers to the gene). RyR1 is a homotetrameric protein and each subunit has a MW of 454 kD, making the tetramer one of the largest known protein molecules in the biological world. The gating properties of RyR1 are affected by several endogenous compounds including ATP, Mg++, Ca++, calmodulin, FKBP, carnosine, NO, and by DHPR. Exactly how RyR1 is regulated during ECC is unknown but the effects of Ca++ and Mg++ have received attention in this regard. Since the main
function of RyR1 is to release calcium from the sarcoplasmic reticulum (SR) stores to the myoplasm, it is fascinating that calcium itself may be regulating the channel. The effects of calcium on channel gating are illustrated by the bell shaped pCa (-log [Ca$^{++}$]) versus Po (open state probability) graph (Figure 4).

Three things are evident from this graph: 1) At a pCa=7, i.e., [Ca$^{++}$] of $10^{-7}$ M and below, the channel is mostly in a closed state and no calcium is released. Stated another way, the channel does not open until the SR extraluminal [Ca$^{++}$] is $>10^{-7}$ M. It is proposed that a high affinity calcium binding site on the myoplasmic region of RyR1 is responsible for opening RyR1 for calcium release. As the [Ca$^{++}$] rises above $10^{-7}$ M to $10^{-6}$ M (1 µM) and then to $10^{-5}$ M (10 µM), the channel Po values increase to a maximum (Figure 5). When the [Ca$^{++}$] ranges from pCa=5 to 4 (10-100 µM) the Po value is maximal. What is not evident from the graph is the existence of an SR intraluminal low affinity calcium binding site that must be occupied in order for the high affinity calcium binding site to open the channel for calcium release [60]; 2) The calcium concentration at which the channel Po is maximal is around $5 \times 10^{-6}$ M. This is evident from the peak Po observed in (Figure 4); and 3) As the [Ca$^{++}$] increases above $10^{-4}$ M, the open state probability of the channel decreases (Figure 4) and around $10^{-3}$M the channel is mostly in a closed state. It has been postulated that yet another low affinity calcium binding site serves to inactivate the channel.

Heat production in skeletal muscle is of metabolic origin and is ultimately traceable to the hydrolysis of ATP. In resting, non-contracted skeletal muscle, heat production may contribute significantly to basal energy production [61]. This skeletal muscle heat production may be the consequence of cross-bridge recycling, ATP-utilizing ion pumps, or any other ATPase mechanism that will result in concomitant heat production. One mechanism considered to be a major source of heat production is calcium ion recycling [62]. In resting muscle there is a 10,000-fold higher concentration of Ca$^{++}$ in the SR lumen versus the myoplasm, and as a consequence of this strong gradient, it is thought that some calcium continuously leaks from the SR storage site and then this calcium is immediately pumped back inside the SR lumen by the SERCA1 calcium pump. SERCA1 utilizes 1 ATP molecule for every 2 Ca$^{++}$ translocated across the SR membrane. A continuance of this “calcium-recycling” by the SR membrane generates heat and contributes to basal energy expenditure. During normal E-C coupling, an action potential in the sarcolemma opens the DHPR channel which is mechanically coupled to and opens the RyR1 channels. The rate of calcium entry into the myoplasm exceeds the rate at which SERCA1 can

---

**Figure 4.** The effects of calcium concentration on the open state probability of single RyR1 channels reconstituted into an artificial lipid bilayer. Single channel recordings were obtained in the presence of cesium (250 mM cis; 50 mM trans) and the open state probability measured after adjusting the pCa level by appropriate additions of EGTA. Data represent single channel recordings from normal pig skeletal muscle sarcoplasmic reticulum membrane vesicles.
pump Ca\(^{++}\) back inside the SR lumen with the net effect being a transient rise in myoplasmic [Ca\(^{++}\)] which activates the contractile elements to produce a contraction. The formation of crossbridges between actin and myosin and the reuptake of released calcium by SERCA1 represents two other E-C coupling events associated with ATPase and heat production. In order to replace the ATP expended for contraction and relaxation, metabolic heat is produced as glucose is utilized for ATP synthesis. Depending on the mitochondrial content of the muscle, glucose will either be converted to lactate or to CO\(_2\) and H\(_2\)O with the concomitant amounts of heat production. When skeletal muscle activity is increased, such as in exercise, a large amount of heat is produced. In a resting state, the average human consumes about 3.5 cc oxygen/kg min\(^{-1}\), and in well trained athletes at maximal exercise, oxygen consumption can increase to 50 cc/kg min\(^{-1}\) and above. This represents a 14-fold increase in oxygen consumption at maximal exercise. In MH, increases in oxygen consumption can be 5-fold that of resting levels which demonstrates the "malignant" metabolism associated with the loss in calcium regulation when no work is being performed.

INVESTIGATIONSOFC[A\(^{++}\)] REGULATIONNMH SKELETAL MUSCLE

The availability of large amounts of skeletal muscle tissue from MHS pigs provided opportunities to investigate the regulation of calcium in intact muscle and in isolated SR membranes under controlled experimental conditions. Initial studies on intact MH muscle measured metabolic responses in MHS versus normal pig muscle. It was observed that after surgical removal of skeletal muscle from the pig, ATP levels declined and lactate levels increased at greater rates in MHS compared with normal pig muscle [63,64]. Subsequently, the in vitro contracture responses of surgically biopsied skeletal muscle to halothane and to caffeine were investigated in MHS skeletal muscle [54,65]. The abnormal responses of MHS pig skeletal muscle were very similar to those observed in MHS human skeletal muscle, providing further confirmation that the MHS pig was a suitable animal model for MH in humans. Furthermore, it was determined that these in vitro contracture responses of MHS skeletal pig muscle could diagnostically distinguish MHS from non-MHS animals [48]. These in vitro pharmacologic responses of MHS muscle were useful for diagnostic purposes but provided only minimal insight into the abnormality specifically responsible for MH.

Methodology for investigation of calcium regulation by isolated SR membranes was very limited in the early 1970s, but a logical target for MH research was anesthetic effects on this membrane system. What was known about SR function at this time was that the calcium pump (SERCA1) was responsible for maintaining the stores of Ca\(^{++}\) within the SR lumen and methods for measuring SERCA1 activity were readily available. Less was known about how calcium was released from the SR, but caffeine was thought to cause release from the SR storage sites. Consequently, the initial studies involved measurements of the calcium uptake ability of MH versus control pig SR membranes. A number of studies measuring calcium uptake in the presence and absence of halothane have been summarized and evaluated [66]. The evidence to date shows no difference in calcium uptake rates between SR from MHS and normal pig skeletal muscle, and that the potent inhaled anesthetics increase the calcium uptake rates in both. This effect of inhaled anesthetics on SERCA1 is opposite that caused by the increases in myoplasmic [Ca\(^{++}\)] occurring in MH. Consequently, it was concluded that the anesthetic-induced MH syndrome could not be explained by the anesthetic effects on SERCA1. Measurements of SERCA1 among unrelated MHS human patients also failed to find an abnormality [66]. The logical conclusion from these studies is that if calcium uptake (i.e., SERCA1) is not the factor in MH calcium dysregulation then calcium release must be involved. While calcium channels were expected to reside in the SR membranes, none had been identified at this time.

With assistance from Dr. Stan Ohnishi we started searching for a method by which we could measure calcium release from isolated SR membrane vesicles and then compare this between SR from MHS and normal pig skeletal muscle. The isolated heavy fraction of SR membranes used in these studies contained the SERCA1 calcium pump and the RyR1 calcium release channel. The cuvette measuring system contained ATP and an ATP-regenerating enzyme/substrate matrix and a calcium-sensitive dye, arsenazo III for measuring [Ca\(^{++}\)] outside the SR membrane. Addition of calcium to the cuvette produced a calcium-arsenazo III absorbance signal that was reversed as SERCA1 pumped calcium into the lumen of the SR (Figure 6). We discovered that if heavy SR membranes were loaded stepwise with fixed amounts of calcium that a critical level of calcium would be reached inside the SR lumen causing release of calcium upon the next addition of calcium (Figure 5). The fact that this release of calcium was followed by a re-uptake of the released calcium suggested channel gating mechanisms. After reaching the critical calcium load threshold (total nmoles Ca\(^{++}\) added per mg SR protein, T1, (Figure 6), adding calcium outside the SR lumen triggered the opening of the calcium release channel. However, as calcium was released, a critical level of calcium outside the SR lumen was thought to close the channel and allow the SERCA1 pump to remove all the calcium released back into the SR lumen. Afterwards, each addition of calcium results in another cycle of calcium-induced calcium release followed by calcium re-uptake. The [Ca\(^{++}\)] on the outside of the SR membrane necessary to open the
Figure 5. Upper Panel: The effects of a clinical concentration of halothane on T1 and T2 for the rabbit SR membrane preparation described for the lower panel. Halothane reduced both T1 and T2 showing that it can increase the sensitivity of the intra- and extra-luminal calcium receptors to calcium. *Represent halothane-induced spontaneous calcium release.

Lower Panel: Rabbit SR membrane vesicles were loaded with calcium inside a cuvette containing MgATP and an enzymatic ATP regenerating system. Serial additions of calcium were followed by uptake of the calcium until a critical amount of calcium was loaded inside the SR lumen. At this calcium load threshold (T1), further additions of calcium resulted in opening the RyR1 calcium release channel with calcium release occurring followed by closure of the channel and return of the calcium to the SR lumen by SERCA1. After determining T1, increasing concentrations of calcium were added until the optimum external concentration of calcium (T2) necessary to open RyR1 was established.
channel (T2), once T1 is reached, can also be determined. After developing this model for studying calcium release from isolated SR membranes, we found that SR from MHS pig muscle had a much lower intraluminal calcium threshold for calcium-induced calcium release [67]. A similar finding was reported by others [68]. These studies provided the initial, critical evidence for a defect in MHS pig muscle involving the processes of calcium release and most likely the calcium release channel itself.

However, the calcium release channel of the SR was not identified until 1988 in Dr. Gerhard Meissner's lab [3]. Designated the ryanodine receptor calcium release channel (RyR1), its biochemical and pharmacologic properties were characterized and one of its distinguishing features was inhibition by ruthenium red. When ruthenium red is added to SR prior to calcium loading, the calcium-induced calcium release is blocked [67], showing that the release mechanisms involve RyR1. Linkage of these two phenomenon, provided an important clue that MHS might be caused by a defect in RyR1. Subsequent studies confirmed the calcium release channel defect in MHS pig skeletal muscle [69-72]. The functional studies on RyR1 provided good evidence that this protein was abnormal in MHS pig muscle and the next step would be to search for a mutation in the gene (RYR1) coding for RyR1. The successful discovery of a mutation in RyR1 in the MHS pig, which occurred in Dr. David MacLennan's lab, will be discussed in detail under the Genetics Section. It is noted here that the MHS pig animal model was responsible for discovery of the key elements in the cause of this catastrophic disease as well as discovery of an efficacious treatment drug.

PROTEINS INVOLVED IN SKELETAL MUSCLE CA++ REGULATION AS POTENTIAL TARGETS FOR MH

Within the E-C coupling pathway are several different protein molecules that function to regulate [Ca++] during contraction, relaxation, and maintenance of the resting state. These proteins shown in (Figure 3), if mutated, could possibly be responsible for increasing calcium cycling and if affected by MH-trigger anesthetics could cause MH.

At present only mutations in genes for RyR1 and DHPR are associated with MHS. The RyR1 calcium release channel is modulated by several endogenous substances including ATP, Ca++, calmodulin, calsequestrin, carnosine, Mg++, and FKBP12.

FKBP12

FKBP12 binds to and modulates the gating properties of RyR1, and the drug FK506, which dissociates to FKBP12 from RyR1, can cause calcium release in normal muscle in the presence of
halothane [73]. We have screened for a mutation in the FKBP12 gene in 14 MHS patients (without RyR1 mutation) and have found none (Nelson, unpublished). If FKBP12 mutations do predispose to MH, they must be rare among the MHS population.

Calsequestrin, Triadin, Junctin

Calsequestrin, a 63 kDa protein with a high capacity and moderate affinity for calcium binding, is concentrated in the SR terminal cisternae. Calsequestrin functions to retain calcium in the terminal sacs of the SR where a larger number of RyR1 calcium release channels reside. This arrangement assures that sufficient calcium is available for release when the RyR1 channels open so that muscle contraction will occur. In addition to sequestering calcium within the SR lumen, calsequestrin may form a complex with RyR1 and regulate RyR1 gating [74]. Because of its key role in intraluminal SR Ca\(^{++}\) homeostasis and a possible role in regulating the RyR1 calcium efflux channel, a mutation in the calsequestrin gene could conceivably predispose to anesthetic-induced MH. A previous study showed no difference between MHS and normal pig calsequestrin for concentration or for Ca\(^{++}\) binding capacity [75]. Halothane can induce oligomerization of calsequestrin in vitro [76] and halothane could possibly have effects on calsequestrin in vivo. At this time no mutations in calsequestrin have been reported in MH susceptible humans or animals. However, a missense mutation in the heart muscle isoform is associated with polymorphic ventricular tachycardia in Bedouin families [77]. Triadin [78] and junctin [79] are very similar compounds found in the terminal cisternae membrane with a major portion within the SR lumen and a minor portion in the cytoplasmic domain. Both molecules contain a KEKE motif (alternating lysine and glutamic acid) providing highly charged regions within the molecules. Triadin may be involved in regulation of calcium release by its interaction with RyR1 [80]. At low Ca\(^{++}\) concentrations (pCa ~ 6) triadin inhibits calcium release from SR vesicles and reduces open state probability of single RyR1 channels incorporated into lipid bilayers [80]. Thus, mutations in either the triadin or junctin genes might alter calcium regulation in such a way as to abnormally sensitize calcium release to the actions of anesthetic agents. When the complex associations between these proteins and calcium release are considered (Figure 3), it is possible that a critical mutation in the gene for any one of these proteins could predispose to MH. Functional studies and drug effects on triadin and junctin are lacking so the interactions between anesthetic action, and calcium regulation by these newly discovered proteins are unknown. To our knowledge, systematic investigation for mutations in the triadin and junctin genes from MH subjects have not been performed.

SERCA1

The skeletal muscle sarcoendoplasmic reticulum CaMgATPase, SERCA1, functions to transport calcium from the myoplasm to the SR lumen, and in so doing it represents the driving force for muscle relaxation. Previous studies [66] have not detected a defect in SERCA1 function in SR from MHS human muscle, but a complete and systematic investigation is lacking. In wild-type SERCA1, volatile anesthetics actually increase rate of calcium uptake [81] which is an effect opposing increased myoplasmic Ca\(^{++}\) and development of the MHS syndrome. However, this does not mean that a mutation in the SERCA1 gene could not produce a reduction in SERCA1 function that could lead to a rise in myoplasmic Ca\(^{++}\) and to MH. Since mutations associated with MHS have been found in only about 50% of affected families, it may be premature to rule out SERCA1 as a target protein for MHS.

DHPR

The nerve-generated action potential propagates along the sarcolemma and down the transverse tubule where it reaches the voltage-gated, dihydropyridine-sensitive calcium channel (DHPR). In skeletal muscle the L-type DHPR is comprised of 4 subunits which include the \(\gamma\) subunit (25 kDa), the \(\beta\) subunit (58 kDa), the \(\alpha_2\) subunit (125 kDa), and the \(\alpha\)-1 subunit (212 kDa). The \(\alpha\)-1 subunit contains the voltage sensor and the Ca\(^{++}\) channel pore. The DHPR transduces the voltage signal to RyR1 by interactions between the II-III loop of DHPR and specific domains of RyR1 [82,83]. This association between DHPR and RyR1 is thought to mechanically open RyR1 and allow calcium to flux from the SR lumen into the myoplasm. Only every other RyR1 is coupled to a DHPR and how these “uncoupled” RyR1 channels are activated has not been determined. The exact DHPR-binding peptide domain(s) of RyR1 have not been determined due to divergence between functional and binding studies. Whether, in addition to the action of DHPR, there are other mechanisms of opening RyR1 (i.e., calcium-induced-calcium release) remains a debatable issue in skeletal muscle. Mutations in the Arg1086 amino acid of the \(\alpha\) subunit of DHPR have been associated with MH [84,85]. In a separate study neither of these DHPR mutations were found among 73 unrelated MHS individuals [9]. The DHPR protein is situated in a prime position in the EC coupling pathway to markedly influence abnormal calcium homeostasis if a critical mutation exists. How significant a role it may play in MH remains to be determined.

RYR1

The ryanodine receptor protein (RyR1) is the major calcium channel through which calcium is
released for skeletal muscle contraction. Teleologically, ryanodine receptors are found throughout the animal kingdom and have structural and functional homology with IP3 receptors. Among mammals, three isoforms have been identified and each appears to have tissue-specific functionality. The RyR proteins are targeted to either the sarco- or endoplasmic reticulum where they function to release Ca\(^{++}\) for activation of a mechanical or biochemical event. RyR1, the predominant isoform in skeletal muscle, is required for skeletal muscle E-C coupling and cannot be replaced by either RyR2 or RyR3. RyR2 on the other hand is the predominant isoform in heart muscle and dictates functional differences in E-C coupling when compared to skeletal muscle. For example, calcium entry via the DHPR receptor is essential to E-C coupling (and to calcium-induced-calcium release) in cardiac but not in skeletal muscle. RyR3 has predominance in neuronal tissue but RyR1 and RyR2 may also be functionally important in some nervous tissue. Only a small segment of RyR1 occupies the SR membrane while a majority of the protein resides in the cytoplasmic space between the SR and the transverse tubular membrane (Figure 3). The exact number of transmembrane segments and how the channel pore is formed is undetermined and estimates range from 4 to 10 transmembrane segments [86]. The functional RyR1 calcium release channel is comprised of four of the 454 kDa subunits and the exact manner in which these subunits interact to produce the channel has not been determined. Such information may be important to MH since most susceptible humans are heterozygous for RYR1 and therefore whether the formation of RyR1 is by random or selected subunit interaction would determine the number mutant subunits present in any given channel. We have previously presented the implications of this in a putative model [87]. Investigations with cryo-electron microscopy have provided a three dimensional structure for RyR1 [88]. RyR1 is a homotetrameric protein and each subunit has a MW of 454 kDa making the tetramer one of the largest known protein molecules in the biological realm. The gating properties of RyR1 are affected by several endogenous compounds which include ATP, Mg\(^{++}\), Ca\(^{++}\), calmodulin, FKBP, carnosine, NO, calsequestrin, triadin, junctin, and the DHPR. Some of these have antithetical actions; low concentrations increasing channel opening while high concentrations block channel opening. In skeletal muscle, every other RyR1 is associated with a DHPR molecule and this represents the linkage mode between depolarization of the sarcolemma and opening of the RyR1 channel.

At the present time, mutations in the RyR1 have been found in about 50% of MHS families studied and >40 different RYR1 mutations associated with MH have been reported (Figure 6). The RYR1 gene located on human chromosome 19 contains 106 exons coding for 5000 amino acids which makes screening for new mutations a very labor-intensive effort. There are three distinct regions of the RyR1 molecule which contain the mutations associated with MH. The C-terminal region of RyR1 contains the SR membrane spanning region with the channel’s pore, and 4 different MH-associated mutations have been reported from amino acids 4637 to 4898 (Figure 6). Another “MH-hot zone” resides in the central region of RyR1 in which 22 different mutations are reported between amino acids 2101 and 2458 (Figure 6). A third region at the N-terminal end of RyR1 contains 12 reported mutations, one of which represents only central core disease (CCD).

**FUNCTIONAL ASSAY OF MUTANT RYR1 PROTEINS EXPRESSED IN NON-MUSCLE CELLS**

Immune Cells: Excitable and non-excitable cells utilize an increase in [Ca\(^{++}\)] as a signal to activate a mechanical or biochemical process. Specialized intracellular organelles and specific proteins are involved transmitting and regulating the Ca\(^{++}\) signal.

A discovery by Sei, and coworkers [89] that B lymphocytes express the skeletal muscle isoform of RyR1 provided a readily available source of RyR1 for investigation. Girard and coworkers [90] capitalized on this finding by investigating Ca\(^{++}\) regulation in virus-immortalized B cells from normal and MH-affected humans. They found that the EC\(_{50}\) concentration of 4-chloro-m-cresol for increase of intracellular [Ca\(^{++}\)] in B cells from normal humans was greater that the EC\(_{50}\) for B cells from MH individuals with a V2168M mutation in RyR1. Since 4-chloro-m-cresol increases the open state of RyR1, the results provide functional data showing that the MH mutant RyR1 in B cells causes a phenotypic response, i.e., increased [Ca\(^{++}\)], consistent with that observed in skeletal muscle.

HEK Cells: The transfection of wild-type and MH-mutant RyR1 cDNA into HEK cells has provided a muscle-independent functional assay for these proteins. This method has been used with success in MacLennan’s lab [91]. In this study, each of 15 MH RyR1 mutations produced abnormal increases in intracellular [Ca\(^{++}\)] when treated with either halothane or caffeine. These functional assays of transfected mutant RyR1 proteins are important conformational data to support but not prove a causal basis for MH. The processes regulating myoplasmic [Ca\(^{++}\)] during skeletal muscle E-C coupling are complex and involve many proteins not present in HEK cells. What role, if any, these ancillary proteins may play in the phenotypic expression of MH in susceptible humans is unknown. Consequently, interpretation of the results from these transfected mutant RyR1 proteins in non-muscle cells should be with guarded optimism.
RYR1 CALCIUM ACTIVATION AND INACTIVATION SITES IN NORMAL AND MH SR

Most of the mutations linking to MH involve RyR1, and consequently it is of significant interest to determine how these mutations predispose to anesthetic-induced increases in myoplasmic [Ca++] and to the MH syndrome. Exactly how RyR1 is regulated during ECC is unknown, but the effects of Ca++ and Mg++ have received attention in this regard. Our initial findings in MH SR membranes [67] indicated that 1) the low affinity calcium inactivation site of the Ca release channel was not affected, 2) the high affinity calcium activation site may or may not be affected, and 3) that an intraluminal, low affinity calcium binding site sustained an abnormally lower calcium threshold for calcium release. Subsequent studies by Fill et al. [71] using reconstituted single RyR1 channels and by Mickelson et al. [70] using radio-labeled ryanodine binding suggested that an abnormality existed in the low affinity calcium inactivation site. In collaboration with Susan Hamilton's lab [92], we failed to find a difference in the high affinity calcium inactivation site between MH and normal pig SR membranes. An important difference exists between the methods used in the two [3H]ryanodine binding studies. Mickelson et al. [70] used different ryanodine concentrations ranging from 10 to 1000 nM, whereas the Hawkes et al. [92] study used a single, 5 nM concentration of ryanodine. It is clear from the Mickelson data [70] that the inability of calcium to inactivate RyR1 is dependent on ryanodine concentration, increasing with each increase in ryanodine concentration (see Figure 1) in Mickelson et al. [70]. The use of [3H]ryanodine to measure the open or closed states of RyR1 is confounded by the actions of ryanodine itself on the channel. Dependent on concentration, temperature, and time, ryanodine can either open or close the channel, and these effects can be occurring at the same time one is attempting to measure the effects of some other agonist or antagonist on the channel. Thus, in the studies by Mickelson et al., the fact that higher concentrations of ryanodine were required to demonstrate abnormal calcium inactivation in RyR1 from MHS muscle suggests that the response to ryanodine effects, not the calcium inactivation site itself, might be producing this observation. In other words, does the requirement for higher [Ca++] for inactivation of the MH RyR1 channel occur in the absence of ryanodine? We have investigated this by measuring single channel activity at pCa levels from 2.0 to 7.5 and find that there is no difference between the calcium inactivation curves between channels from MHS and normal pig muscle (Figure 5). Thus, measuring single channel activity in the absence of ryanodine our data argue against an abnormality in the high affinity calcium inactivation receptor of RyR in MHS pig muscle.

Figure 7. Comparison of the calcium-activation and -inactivation curves for RyR1 channels from MH susceptible (MHS) and normal (MHN) pig skeletal muscle. Experiments were performed using cesium (250 mM cis/50 mM trans) as the conducting ion and calcium was buffered with EGTA to obtain the different pCa levels.
HOW DO TRIGGER ANESTHETICS AFFECT MUTANT RYR1 CALCIUM RELEASE CHANNELS?

Many of the studies on RYR1 have utilized naturally occurring ligands such as ATP, Ca++, calmodulin, etc., and drugs such as ryanodine, caffeine, and 4-chloro-m-cresol (4CmC). Caffeine, ryanodine, and 4-CmC have been used as diagnostic tools for MH in measuring the in vitro contracture response of freshly biopsied skeletal muscle. However, none of these are considered to be causal agents for MH, and yet few studies have been done to measure the effects of MH trigger anesthetics on RYR1 to explain how they cause the MH syndrome. Succinylcholine acts on the postsynaptic receptors to produce transient muscle paralysis by depolarization of the sarcolemmal membrane. This brief succinylcholine-induced depolarization also opens the DHPR voltage gated calcium channels, which in turn act on the RYR1 to cause calcium release invoking the same mechanisms produced by acetylcholine release. Prior to muscle paralysis it is not uncommon to observe skeletal muscle fasciculations following the administration of succinylcholine. How this depolarizing signal from succinylcholine triggers MH is not entirely known, but succinylcholine does not readily cross membranes and therefore a direct action on the mutant RYR1 calcium release channel or other E-C coupling proteins inside the muscle cell would not be expected. It is clearly established that, following burns or major denervation phenomenon, there is proliferation of acetylcholine receptors on the sarcolemma, making the patient susceptible to succinylcholine-induced hyperkalemic rhabdomyolysis [93]. A number of cases of succinylcholine-induced hyperkalemic mortalities in young males (likely carriers of DMD) has resulted in a recommendation that its use in children be only in emergent situations. It may be possible that MH causes a proliferation of acetylcholine receptors, which in turn causes an abnormally high signal input to the mutant RYR1 calcium channels leading to larger amounts of calcium release that triggers MH. Another explanation is that the normal amount of succinylcholine-induced signal entering the MH muscle is sufficient to trigger calcium-induced calcium release that cascades into the MH syndrome. The preservatives used in formulating succinylcholine can open RyR1 channels, but the concentrations required to produce this effect are much higher than those obtained during clinical use of this drug [94], making the preservatives an unlikely suspect in this syndrome.

The potent, inhaled volatile anesthetic agents are unquestionably the triggers for MH in genetically predisposed individuals. These lipophilic molecules readily cross membranes and access intracellular proteins. Clinically, these potent inhaled anesthetics contribute to muscle relaxation, but whether this is a central or peripheral nerve effect or an effect on skeletal muscle or a combination of these is unknown. It is of interest to note that directly stimulated, isolated skeletal muscle contractility is potentiated by the volatile agents in vitro [95]. Experiments are needed to determine what in vivo effect the potent inhaled anesthetics have on contractility of the directly stimulated skeletal muscle. It is important to determine if the volatile anesthetics do potentiate contractions in normal skeletal muscle in vivo because this might relate to the mechanisms by which they trigger the MH syndrome during clinical anesthesia. The in vitro data certainly suggest that the twitch potentiating effects of volatile anesthetics in normal muscle in vitro is converted to an isometric contracture producing effect in MH muscle (Figure 8). Data published from our laboratory support the theory that volatile anesthetics act directly on RyR1 to increase the open state probability and to prolong the time the channel dwells in an open state [96]. The effect of this action would be to increase the rate of calcium release from the SR stores with the net effect being an increase in myoplasmic calcium. The metabolic consequences of this increased myoplasmic [Ca++] will be discussed below. Our initial studies [67,81] utilized isolated SR membranes and measured the effects of volatile anesthetics on calcium uptake (SERCA1) and on calcium release (RyR1). We found that potent volatile anesthetics increase the rate of calcium uptake by SERCA1 [81], which would tend to protect against MH by lowering the myoplasmic calcium concentration. Subsequent studies by other laboratories have confirmed these findings [97]. However, the effect of volatile anesthetics in increasing calcium release from the SR [81,97] and increasing the open state probability of RyR1 single channels [96] are in a direction that is consistent with the contractile potentiating effects in normal muscle and the contracture-producing effects in MH muscle. The latter effect in MH muscle is associated with development of the MH syndrome.

In the calcium uptake and release SR model one can measure the essential amount of calcium that must be loaded before calcium-induced calcium release can be observed. This threshold amount of calcium load for calcium release is referred to as Threshold I (T1) and is expressed as nmoles Ca per mg SR protein. We postulated that the SR membrane contained an intraluminal, low affinity calcium receptor that is activated by a critical (i.e., T1) concentration of intraluminal calcium [60]. Once the low affinity calcium receptor is activated, then an extraluminal, high affinity calcium receptor can be activated to open the RyR1 channel and release calcium. We have found that the volatile anesthetics lower T1 and thus allow calcium release to occur at lower T1 levels (Figure 6). This effect of the anesthetics occurs both in normal and in MHS membranes but is more profound in membranes from MHS animals. In SR from MH pig and dog skeletal muscle, T1 is much lower than normal both in the presence and in the absence of volatile anesthetics. In view of the current models for the spatial
arrangements for RyR1, there is insufficient information to correlate the abnormal intraluminal calcium receptor response in MH muscle with the occurrence of RyR1 mutations in the cytoplasmic region. In other words, how do the mutations in the cytoplasmic regions of RyR1 in the pig (amino acid # 614) and in the dog (amino acid # 547) cause an alteration in the low affinity calcium binding site inside the SR lumen? How the cytoplasmic region of RyR1 interacts with and regulates the pore forming region and the gating properties of the RyR1 calcium channel is unknown. Since intact skeletal muscle does not require extracellular calcium for E-C coupling then the contribution of calcium via the voltage-gated calcium channel to CICR is not essential in skeletal muscle as it is for cardiac muscle. This has led to the theory that the voltage-gated DHPR channel is physically attached to the cytoplasmic region of RyR1 and through this attachment the DHPR responds to membrane depolarization in a way that causes a conformational changes in the cytoplasmic domains of RyR1 and these changes are translated “long-range” across the molecule to open the channel gate. In MH, mutations in the cytoplasmic region may have altered these putative long-range allosteric interactions [98] in a manner that results in increased open state probability and increased open dwell-times of RyR1 which produces abnormal calcium release. Excellent studies by Ikemoto and collaborators [99,100] have provided evidence for RyR1 regulation by RyR1 subunit interdomain interactions. The data support a hypothesis that a peptide region of the N-terminal domain (amino acids 590-628) interacts with a region of the central domain (amino acids 2442-2477) to stabilize the conformational “off state” (i.e., closed state) of RyR1. Since many of the RYR1 mutations linking to MH fall within either the N-terminal or central domains, these investigators propose that the mutations destabilize the interdomain interactions maintaining the “off-state,” making a transition from the closed to the open state more probable. Furthermore, such destabilization is proposed to hypersensitize RyR1 to drugs that act to open the channel. The regulation of RyR1 is very complex, and it will be interesting to see if these important studies reflect a mechanism of RyR1 regulation and an explanation for how RyR1 mutations cause MH. If this system is perturbed by a pharmacologic agent in a way that augments the effect of the MH mutation, then larger amounts of calcium will be released within the muscle cell and the MH syndrome will develop.

**DIAGNOSTIC SCREENING FOR MH**

The inheritance of MH in the majority of families as an autosomal dominant trait is illustrated by the pedigree (Figure 9). Consequently, identification of an MH proband by a clinical episode raises concerns about the disease among the individual’s relatives.
Figure 9. *In vitro* caffeine-induced isometric contracture response in skeletal muscle from normal (MHN) and malignant hyperthermia susceptible (MHS) human skeletal muscle. The curves show part of the caffeine concentrations used to distinguish between MHS and normal skeletal muscle. The horizontal (1.0 g) and vertical (concentration) lines depict the method used to calculate the caffeine specific concentration (CSC) required to produce 1.0 g of isometric contracture. The MHS muscle CSC is about 2.5 mM versus the normal muscle value of 5.0 mM. Data are derived from patients tested at the Wake Forest University School of Medicine's MH Diagnostic Center.

For the past 27 years, the only reliable method for diagnosing MH has been the measurement of isometric contracture responses of freshly biopsied skeletal muscle to caffeine and to halothane. Protocols for MH diagnostic contracture testing of human skeletal muscle have been developed by the North American [101] and European [102] MH Groups. The caffeine contracture test was derived from the early and important discovery by Kalow and Britt that muscle obtained from MH survivors had abnormal contracture responses to caffeine and that halothane potentiated these responses [15]. Caffeine had been used for years by pharmacologists to produce skeletal muscle contracture at high concentrations or to potentiate electrically-evoked contractions at lower concentrations. Caffeine, by some unknown mechanism at that time, was creating an increase in myoplasmic calcium and this produced the sustained contractures. It is now known that caffeine acts on the ryanodine receptor calcium release channel to increase open state probability of the channel allowing calcium to flow from the sarcoplasmic reticulum stores into the myoplasm. The rate at which caffeine causes calcium release exceeds the rate at which the sarcoplasmic reticulum calcium pump (SERCA1) can return calcium to the SR lumen and this results in a net increase in myoplasmic calcium. The concentrations of caffeine that only potentiate the electrically-evoked twitch in normal muscle produce contracture in MH skeletal muscle. In MH skeletal muscle the caffeine dose versus contracture tension response curve is shifted to the left of the curve for normal muscle (Figure 3). An important discovery by Ellis et al. [16] that halothane alone produced contracture in MH muscle was also a breakthrough not only as another diagnostic tool, but because halothane, not caffeine, is what causes MH in humans. Thus, the fact that halothane also produces contracture in isolated skeletal muscle was evidence that this was the primary tissue in which anesthetics triggered the MH syndrome. In normal muscle halothane produces little or no contracture at all and only potentiates the electrically-evoked twitch response (Figure 10 Upper Panel). In MH muscle halothane produces a large isometric contracture response (Figure 10 Lower Panel), which is thought to mimic what halothane does to MH muscle in vivo. The *in vitro* contracture phenotyping of MH and normal skeletal muscle is important not only to the diagnosis of individuals, but is also important for establishing lod scores for confirming linkage of MH.
Figure 10. Upper Panel: The *in vitro* exposure of normal human skeletal muscle to halothane, 3% results in potentiation of the electrically-evoked twitch response with no change in baseline tension.

Lower Panel: In MHS muscle the exposure to halothane, 3% results in an isometric contracture that is diagnostically characteristic for MHS skeletal muscle.
susceptibility to altered genes. Like all diagnostic tests, the MH contracture test is not perfect, and by the North American MH Protocol standards, the sensitivity and specificity are 97-99% and 85-90%, respectively. The European protocol represents sensitivity and specificity values of 100% and 90%, respectively.

It is now clearly established that MH is genetically heterogeneous in humans while single point mutations in the ryanodine receptor gene in pigs [6] and in dogs [7] link to MH in these species. In spite of this, there is remarkable contracture phenotype similarity across species. We have reported previously [87] that MH humans, pigs and dogs all have abnormal in vitro muscle contracture responses to caffeine, halothane, and to ryanodine. Ryanodine is a plant alkaloid that binds avidly to RyR1 and can cause a prolonged open state of the channel. Each of these drugs has been shown to increase the open state probability of the ryanodine receptor calcium release channel after it is reconstituted into an artificial lipid bilayer. This action may explain how calcium is increased when skeletal muscle is treated with one of these drugs. What is clear is that across, and within, a species different mutations in RYR1 lead to the same phenotype which is abnormal contracture sensitivity to halothane, caffeine, or to ryanodine—the hallmark of MH skeletal muscle. Our laboratory has utilized 3 different tests to phenotype human, pig, and dog skeletal muscle. In addition to the standard halothane and caffeine tests, we have added a test in which caffeine and halothane are combined [103]. In the two caffeine contracture protocols, one with and one without halothane, we have calculated the caffeine specific concentration (CSC and HCSC) necessary to produce 1 g isometric contracture response. Three dimensional plots of the CSC, HCSC, and halothane contracture values have been compared among the three species studied (Figure 11). What is evident from these graphs is that the more homogeneous the species (pig>dog>human) the less spread there is in either the MHS or MHN values. The pigs used in our studies were purebred Poland China, and the dogs all had a common sire, while the humans were obviously more heterogeneous in genetic background. But even within the porcine species, a wide spread in contracture sensitivity can be observed among offspring from the same sire and dam, suggesting that genetic influence on the contracture sensitivity of skeletal muscle to these drugs is not simple and straightforward. When the caffeine sensitivity (expressed as CSC values) is compared among 475 human MH diagnostic subjects (both positive and negative results) a wide spread in values from <2 mM to 26 mM caffeine is observed (Figure 12). Whereas the CSC averages about 5 mM for normal human muscle, some individuals have very caffeine-insensitive muscle with CSC values 3 to 5 times higher than the average.

![Figure 11. Three-dimension plot of normal (MHN) and malignant hyperthermia susceptible (MHS) skeletal muscle in vitro contracture responses to halothane, 3%, to caffeine (CSC), and to caffeine in the presence of halothane, 1% (HCSC). Data from three different species show greater homogeneity in these contracture responses for pigs>dogs>humans.](image)
Figure 12. Distribution of the caffeine concentrations required to produce 1 g isometric contracture in skeletal muscle from MHS and normal human subjects. All values <4 mM are attributed to MHS muscle and all values ≥4 mM are attributed to normal muscle responses. The average response for MHN muscle is 5 mM while MHN values range from 4 to 26 mM.

GENETIC SCREENING FOR MH

The ideal clinical screening test will have very high sensitivity (the test will be positive in people with disease) and very high specificity (independent probability that the test will be negative in individuals without the disease).

Unfortunately, MH has two intrinsic features that adversely affect resolution of genetic screening for MH. The inheritance of MH is heterogenous in that several different variants of the same RYR1 gene (allelic diversity) and variants of different (DHPR) genes (locus heterogeneity) are associated with MHS. The presence of heterogeneity in a genetic trait reduces the clinical sensitivity. The other factor affecting resolution of MH genetics is variable penetrance. The probability that MH will occur when an individual with a given MH genotype is exposed to anesthetic agents is not 100%, and therefore the occurrence of clinical MH is affected by other genetic and environmental factors. Consequently, MH can be inherited with incomplete penetrance. Incomplete penetrance in MH will reduce the predictive value of genetic testing. About 50% of MH families investigated to date have point mutations in the RYR1 gene associated with MH susceptibility. In some of these mutations the number of individuals and families is sufficient to support linkage, and guidelines for using RYR1 screening for diagnostic purposes have been published.

GENOTYPE/PHENOTYPE RELATIONSHIPS IN MH

The “catch 22” in human MH genetics is how discordance [104-107] is resolved when genetic investigation depends upon the imperfect contracture phenotyping of MH individuals. Clinical episodes in which signs of MH have resulted in therapeutic and other interventions do not always prove MH susceptibility in an individual. A clinical grading scale [108] was established by an international panel of experts in order to estimate the likelihood that a given clinical episode represented MH susceptibility in that patient. While this grading scale is helpful in evaluating probands, it is seldom used for families because in recent times it has become uncommon for more than one family member to experience MH. Consequently, phenotyping for genetic investigation has become almost completely dependent upon in vitro pharmacologic contracture testing of freshly biopsied skeletal muscle. Both the North American [101] and European [102] MH testing protocols utilize in vitro
muscle contracture responses to caffeine and to halothane for MH phenotyping. While differences exist between the methods of applying these drugs for MH testing, published studies [109,110] suggest that the outcomes are the same regardless of which protocol is used. An exception to this is the fact that the European protocol generates an MH equivocal group if only one of the two tests has a positive result. In contrast, the American protocol requires an abnormal response by only one in three fascicles within any test for a positive result. With the appearance of potential genetic markers for MH, how will the dilemma of genotype/phenotype discordance be resolved and what will be the criteria by which genetic screening replaces in vitro pharmacologic contracture testing? The amplification of calcium release in MH skeletal muscle by anesthetic agents is the hallmark for this disease. In spite of the fact that more than 30 different single point mutations in the RYR1 have linked to MH, each of these mutations leads to an abnormal in vitro skeletal muscle contracture response to various drugs including potent inhaled anesthetics, caffeine, ryanodine, and 4-chloro-m-cresol. Regardless of MHS species or which mutation is causing MH, dantrolene, which acts to block calcium release from RyR1, is therapeutically efficacious for the anesthetic syndrome. This suggests that the defect causing MH is either at or proximal to RyR1 in the EC coupling chain of events. Taken together, these findings provide a basis for expecting the in vitro contracture phenotype to identify the MH genotype but not define the specific mutation linking to MH. The fact that these pharmacologic in vitro muscle contractures are able to identify MHS subjects is remarkable when one considers the complexities of E-C coupling in general and those of RyR1 regulation specifically.

CONCLUSION

The final major barrier to management of MH is the development of a simple, noninvasive diagnostic test that will identify all MHS families and individuals. This will be possible when all mutations predisposing to MH are identified. The rapidly developing technologies in sequencing large genes will shorten the time for discovery of all MH-related genetic defects. In the meantime, anesthesia providers must remain vigilant and prepared, muscle contracture testing must continue, and more geneticists must get involved in resolving all the mutations predisposing to MH.

REFERENCES


A natural text representation of the document is not provided as the text is not clearly visible or legible in the image.