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Review

Mechanistic models for muscle diseases and disorders originating in the sarcoplasmic reticulum☆

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ABSTRACT

This review focuses on muscle disorders and diseases caused by defects in the Ca^{2+} release channels of the sarcoplasmic reticulum, the ryanodine receptors, and in the luminal, low affinity, high capacity Ca^{2+}-binding proteins, calsequestrins. It provides a time line over the past half century of the highlights of research on malignant hyperthermia (MH), central core disease (CCD) and catecholaminergic polymorphic ventricular tachycardia (CPVT), that resulted in the identification of the ryanodine receptor (*RYR*), calsequestrin (*CASQ*) and dihydropyridine receptor (CACNA1S) genes as sites of disease-causing mutations. This is followed by a description of approaches to functional analysis of the effects of disease-causing mutations on protein function, focusing on studies of how mutations affect spontaneous (store overload-induced) Ca^{2+}-release from the sarcoplasmic reticulum, the underlying cause of MH and CPVT. Subsequent sections describe results obtained by analysis of knockin mouse lines carrying MH- and CCD-causing mutations, including a *Casq1* knockout. The review concludes with the presentation of two mechanistic models. The first shows how dysregulation of Ca^{2+} homeostasis can lead to muscle diseases involving both RyR and Casq proteins. The second describes a theory of central core formation wherein non-uniformity of Ca^{2+} release, resulting in non-uniformity of muscle contraction, is presented as an intrinsic property of the specific tertiary structure of mutant heterotetrameric ryanodine receptors and as the underlying cause of core formation in skeletal muscle. This article is part of a Special Issue entitled: 11th European Symposium on Calcium.

1. Introduction

Dr. Witold Drabikowski was a pioneer in the investigation of proteins of the skeletal muscle I-filament, who made many early contributions to our understanding of actin polymerization, its interaction with tropomyosin and the Ca^{2+} binding properties of troponin. In about 1970, he became interested in proteins of the sarcoplasmic reticulum and trained a series of students in that area. To my good fortune, he then sent Drs. Elzbieta Zubrzycka, Marek Michalak and Bozena Korczak to my laboratory for postdoctoral work. Each of these talented, well-trained individuals made unique and lasting contributions while working in my lab and in their later studies. For example, Elzbieta advanced muscle cell culture and cell biology in our lab [1] and later, in Dr. Ron Worton's lab, she made antibodies to dystrophin, which, in mutant form, causes Duchenne muscular dystrophy. With Drs. Worton and George Karpati, she was the first to describe the association of dystrophin with sarcolemmal vesicles [2,3]. Marek has defined the many interesting properties of the ER luminal chaperone protein, calreticulin [4,5]. Bozena was deeply involved in the first cloning of SERCA cDNA and *ATP2A* genomic DNA [6,7].

As we meet to celebrate the life of Dr. Witold Drabikowski, we look back to the discoveries that were being made during his lifetime and think about progress that has been made since then in the fields that were of interest to him. The developments that we will review have taken place since about 1960. They concern muscle disorders and diseases caused jointly by defects in the Ca^{2+} release channels of the sarcoplasmic reticulum, commonly referred to as ryanodine receptors (RyR) – the skeletal muscle RyR1 isoform encoded by the *RYR1* gene on human chromosome 19q13.1, and the cardiac RyR2 isoform encoded by the *RYR2* gene on human chromosome 1q42.1–43 – and by defects in the luminal, low affinity, high capacity, Ca^{2+} binding/buffering protein referred to as calsequestrin (Casq) — skeletal muscle Casq1, encoded by the *CASQ1* gene on human chromosome 1q21 and cardiac Casq2, encoded by *CASQ2* on human chromosome 1p13.3–p11. In early sections, we will provide a time line over the past half century of the highlights of research on malignant hyperthermia (MH), central core disease (CCD) and other core myopathies and catecholaminergic polymorphic ventricular tachycardia (CPVT) and, in the concluding sections, we will offer unifying theories for the etiology and the pathogenesis of all of these muscle disorders and diseases.

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2. Genetic diseases relating to the sarcoplasmic reticulum

2.1. Malignant hyperthermia

The year 2010 marks the 50th anniversary of Dr. Michael Denborough's groundbreaking description of an episode of malignant hyperthermia (MH) in a patient with a family history of anesthetic-related deaths [8]. Forewarned, Dr. Denborough recognized the initial stages of the MH episode and was able to intervene successfully, saving the patient from certain death from this dreaded side effect of surgery under anesthesia. Because of his insights and his personal efforts to publicize the disorder [9,10], MH would, henceforth, be viewed as an inherited disorder that could be studied, understood and prevented.

MH (MH; MIM# 145600) is a subclinical, autosomal dominant pharmacogenetic disorder of skeletal muscle caused by defective intracellular Ca^{2+} homeostasis and presenting with a fulminant and potentially lethal hypermetabolic reaction to inhalational anesthetics and the depolarizing muscle relaxant, succinylcholine [11,12]. The incidence of an MH episode is 1 in 60,000 adults and 1 in 15,000 children and adolescents undergoing anesthesia [13,14]. However, recent estimates are that the actual proportion of individuals, carrying the MH trait, is as high as 1 in 2000 [15]. Genetic testing has shown that about 70% of MH patients have defects in the *RYR1* gene (MIM 180 901) [12,16,17], while a few MH patients have mutations in the *CACNA1S* gene (MIM 114 208) [15,18–22] encoding the α1-subunit of the skeletal muscle voltage-dependent L-type Ca^{2+} channel (Cav1.1), located in the transverse tubular membrane.

An anesthetic-induced MH episode is manifested as a rapid and uncontrolled rise in myoplasmic Ca^{2+}, leading to sustained skeletal muscle rigidity and hypermetabolism and accompanied by a critical drop in ATP levels, acidosis, hypercapnia, tachycardia and an abnormal rise in body temperature [16]. Many of these signs and symptoms are monitored routinely during anesthesia in most modern hospitals and a rapid change in any of these parameters calls for immediate cessation of anesthesia and administration of dantrolene, a potent antidote to anesthetic-induced MH [23–25]. Such precautions reduced fatality from MH from over 80% to less than 10% in the 1990s, but recent surveys on the occurrence of MH in North American clinical institutions show alarming statistics — a rise in MH-associated morbidity to 35% [24] and a rise in MH mortality to 14% [26] during the first decade of the 21st century, presumably because of the use of MH-triggering anesthetics outside of conventional hospital settings [27]. This highlights the need for more comprehensive genetic testing that would alert physicians to the MH susceptibility (MHS) status of patients and the need for a better understanding of the etiology and pathophysiology of the underlying MH defect.

2.2. Central core disease (CCD) and related congenital myopathies

Central core disease (CCD, OMIM #117,000) was first described in 1956 as a rare, non-progressive congenital myopathy, characterized by hypotonia and proximal muscle weakness and presenting with well-delineated areas of myofibrillar disorganization (cores) within myofibers [28]. Additional variable clinical features include *pes cavus*, kyphoscoliosis, foot deformities, congenital hip dislocation and joint contracture [29]. Although clinical signs of CCD may be severe, up to 40% of individuals demonstrating central cores may be asymptomatic [30,31] and lead lives requiring considerable physical exertion.

In 1960, Dubowitz and Pearse [32] described histochemical studies showing that "core" regions within the myofiber of CCD patients did not react with stains for phosphorylase and oxidative enzyme activity. The diagnosis of CCD has since been made on the basis of skeletal muscle weakness and the lack of oxidative enzyme activity in the central regions of skeletal myofibers. In 1961, Engel et al. [33] showed that the cores lacked mitochondria and a well-organized sarcotubular system and that myofibrillar organization was disrupted. Electron microscopic analysis of the cores showed disintegration of the contractile apparatus, ranging from blurring and streaming of the Z lines to total loss of myofibrillar structure [34,35]. The sarcoplasmic reticulum and transverse tubular systems were increased in content and were, in general, less well structured. Mitochondria were depleted in the cores, but may have been enriched around the surfaces of the cores, while retaining normal structural organization.

In 1973, Denborough et al. [36] showed a close association of CCD with MH susceptibility. This association had important genetic consequences because it prompted studies of the genetic basis of MH to be carried out in parallel with studies of the genetic basis of CCD. Some mutations in the *RYR1* gene have been associated with both MHS and slowly progressive skeletal muscle weakness and cores in some, but not necessarily all, MHS members within a family, giving rise to the diagnosis of MH/CCD [17,37]. Some investigators [38–41] have argued that an MH/CCD diagnosis for cases of MH that present with cores may not be valid in the absence of muscle weakness. Indeed, detailed histopathological examinations show that MH is an asymptomatic myopathy and the presence of cores in myofibers does not necessarily mean CCD, since the development of cores can be non-specific and is observed in many cases of muscle injury unrelated to CCD [33,42–44]. Thus it has been suggested that MH cases presenting with cores in the absence of muscle weakness might be more appropriately referred to as MH with cores and not MH/CCD [38,40]. Other RyR1-related myopathies are multiminicore disease (MmD) [45], MmD with external ophthalmoplegia (OMIM #255320) [46], congenital myopathy with cores and rods [47,48], central nuclear myopathy [49], neuromuscular disease with uniform type 1 fibers [50] and congenital fiber type disproportion [51]. For all of these disorders, Ca^{2+} dysregulation is likely to be a primary cause and a common etiology is possible.

2.3. Catecholaminergic polymorphic ventricular tachycardia (CPVT)

CPVT is a congenital arrhythmogenic cardiac disorder that was first described in 1978 [52], with a follow-up report in 1995 [53]. It manifests in apparently healthy juveniles or young adults as syncopal events (blackouts) or, occasionally, with sudden cardiac death as the first clinical indicator of the disease. As with an MH reaction, an arrhythmogenic episode can be fatal if undiagnosed or untreated, but can be managed if diagnosed early. Arrhythmogenic events are usually triggered in response to intensive exercise or emotional stress [54–56]. Estimates of the contribution of CPVT to the incidence of sudden death range from 15% to 30%.

The diagnosis of CPVT is difficult because cardiac evaluation, even when supplemented with echocardiograms and electrocardiograms, often fails to detect any defect. CPVT is suspected when exercise stress tests show isolated premature beats at the beginning of exercise, with a progressive worsening of the complexity of ventricular arrhythmias in response to an increased workload [52,57]. Bidirectional ventricular tachycardia (VT), which is characterized by a 180° beat-to-beat rotation of the axis of the QRS complexes on the frontal plane, is considered the diagnostic marker of CPVT, but not all patients manifest this form of arrhythmia.

Investigations of the genetic basis of CPVT [57–60] showed that it was caused, in part, by dominant mutations in *RYR2*, the cardiac ryanodine receptor (RyR2) gene [57], and, in part, by recessive mutations in *CASQ2* [60] that cause loss, truncation and/or misfolding of cardiac calsequestrin (Casq 2) [61–65]. The incidence of causal *RYR2* mutations is at least an order of magnitude higher than the incidence of causal *CASQ2* mutations. The fact that the CPVT phenotype is almost identical, regardless of whether the causal gene is *RYR2* or *CASQ2*, in both humans and in mice [61–65], suggests that the causal mechanism is the same.

A critical feature of CPVT is that triggering of an arrhythmogenic episode occurs in the absence of structural alterations to the heart and requires the presence of both a causal mutation and stressful conditions that induce β-adrenergic stimulation. Indeed, CPVT responds to β-blockers [66,67]. This is highly analogous to MH in which triggering of an MH reaction, in the absence of any structural alteration of the skeletal muscle, requires the presence of both a causal mutation and either a triggering anesthetic or, in the case of pigs, stressful conditions.

3. A time-line of major advances in MH, CCD and CPVT research

3.1. Ca^{2+} regulation

Early investigations of MH [8,9,68] and CCD [28,69] coincided with other relevant advances in muscle research. The most important of these was the rediscovery, after a hiatus of knowledge lasting several decades, that skeletal muscle contraction requires Ca^{2+} [70–75]. A beautifully reasoned review from this time by Dr. Setsuro Ebashi [76] describes robust experimental evidence showing that Ca^{2+} regulation of skeletal muscle contraction occurs within a complex of actin, myosin, tropomyosin (Tm) and the troponin (Tn) complex, a topic that was of great interest to Dr. Witold Drabikowski. Binding of Ca^{2+} with high affinity to the TnC component of the Tn complex initiates complex conformational changes in both Tn and Tm that shift the location of the TN–Tm complex, permitting contractile interactions between actin and myosin that were precluded by the structure of the Tn–TM–actin complex in the absence of Ca^{2+} [77]. Thus it was apparent to MH researchers that the contracture induced in an MH reaction would, inevitably, require elevation of regulatory Ca^{2+} in those skeletal muscles involved in an MH episode.

3.2. Metabolic regulation

In 1968, Dr. Edwin Krebs' group discovered a cAMP-dependent protein kinase (PKA) that phosphorylates and activates phosphorylase kinase [78]. In 1971, the same group demonstrated that activation of phosphorylase kinase to phosphorylate and activate phosphorylase also requires Ca^{2+} (one subunit of phosphorylase kinase is the Ca^{2+} binding and regulatory protein, calmodulin) [79]. These discoveries led to understanding of the key, coordinated roles of cAMP, PKA and Ca^{2+} in adrenergic signaling pathways in muscle [80] and, since glycogenolysis, catalyzed by activated phosphorylase, is the first step in glycolysis, MH researchers immediately understood that adrenergic pathways must be involved in the elevation of anaerobic metabolism (hypermetabolism) and the increase in blood glucose that were observed during an MH episode.

3.3. Pig model of MH

By the mid-1960s, Hall [81] and Harrison [82] recognized that individual animals in certain lines of pigs were sensitive to volatile anesthetics. Homozygous MH pigs, when subjected to a short, controlled exposure to halothane (the halothane challenge test), developed symptoms characteristic of an MH episode seen in heterozygous MHS humans exposed to halothane, often in the presence of succinylcholine [83]. Fortuitously, breeders selecting for pigs with large hams and lean muscle mass had embedded a single autosomal *HAL* gene (the MH gene) into an exceptionally useful animal model for MH, since pigs and humans have a comparable body size [16]. In addition to the apparent differences in MH inheritance – recessive in pigs, but dominant in humans – there were other apparent differences between MH presentation in pigs and in affected humans. For example, an MH episode is frequently triggered in pigs by exercise, overheating, mating or by emotional stress induced during shipping to the slaughterhouse, so that, in those days, 1–2% of pigs died before reaching market. It was thus clear that an adrenergic trigger was driving what was known as the porcine stress syndrome (PSS) [83,84]. Moreover, the MH episode did not cease after slaughter, but continued in cuts of meat on the packaging line, giving rise to pale, soft, exudative (PSE) pork [85,86].

3.4. Metabolic and ionic changes in MH

In 1970, Dr. Mervyn Berman examined a range of changes in metabolism and ionic composition in pigs following exposure to halothane [87]. He noted a shift of water into the intracellular space, so that serum Na^+ and total protein concentration were elevated, together with a shift of Ca^{2+} and Mg^{2+} into the extracellular space. He also observed: release of PO_4^{3-} into the plasma; an elevation in blood glucose; an uncompensated metabolic acidosis, resulting entirely from a rise in plasma lactate; lowered arterial pO_2 and elevated pCO_2. These biochemical changes preceded temperature elevation and were not associated with heat injury. The rise in blood glucose and excess lactate production resulted from uncontrolled glycogenolysis. Anaerobic sources of heat included glycolysis, the neutralization of lactic acid and the hydrolysis of high-energy phosphate esters (*e.g.* ATP). Fever was not caused by oxidative mechanisms or by the uncoupling of oxidative phosphorylation. The observed rise in muscle protein concentrations in the blood – chiefly myoglobin and creatine kinase – indicated muscle fiber damage. The release of Ca^{2+} from myofibers supported the role of abnormal Ca^{2+} homeostasis in MH, but it was the elevation of blood K^+ levels from 4 mM to 6–7 mM that was most critical, since these concentrations of K^+ in the serum cause cardiac arrest in humans. Hyperthermia is a relatively late sign — it develops 10–15 min after the onset of the reaction [16,83,87].

3.5. Primary site of the MH reaction

In addition to the question of whether the MH defect lay in regulatory Ca^{2+} levels, it became important to early MH researchers to know whether the site of the defect was within organelles inside myofibers or in electrically excitable membranes of the nerves or the sarcolemma. In the early 1960s, Ebashi [71,73,74] and Hasselbach [88,89] established that isolated sarcoplasmic reticulum (SR) vesicles can take up Ca^{2+} in substantial amounts, demonstrating that the "Marsh relaxing factor" [90] is a preparation of SR vesicles. At this time, caffeine was found to induce skeletal muscle contracture and to release Ca^{2+} as a result of the direct action of the drug on the sarcoplasmic reticulum [91]. Thus, from these early times, the sarcoplasmic reticulum was recognized as the probable site of the MH defect and moreover, caffeine became the first important drug for investigation of the primary site of the MH reaction [92].

In equally important studies from this time, Dr. Makoto Endo showed that Ca^{2+} release from the skeletal muscle SR is regulated by a high affinity Ca^{2+} sensor site on the cytosolic surface of the Ca^{2+} release channel so that a rise in cytosolic Ca^{2+} to 1 μM or higher would trigger further Ca^{2+} release in a process that he termed Ca^{2+}-induced Ca^{2+} release (CICR) [93]. Dr. Alexandre Fabiato later demonstrated that excitation–contraction (EC) coupling in cardiac muscle is mediated by CICR [94].

3.6. In vitro diagnostic tests for MH

The first to take advantage of the knowledge that caffeine might be a useful tool in MH research was the team of Dr. Werner Kalow, often described as the "father of pharmacogenetics", and Dr. Beverley Britt, an anesthesiologist devoted to unraveling the biochemical and genetic bases for MH and to the development of preoperative diagnosis of MHS. In an ingenious synthesis of talents and ideas, they isolated muscle fibers from MHS and normal patients and measured the development of contracture of fibers, mounted on a

transducer, in response to increments of caffeine [95]. They noted that tension developed in MHS fibers at lower caffeine concentrations than normal. Shortly thereafter, Richard Ellis established, using the Kalow–Britt approach, that MHS fibers are also hypersensitive to halothane [96]. Thus the North American caffeine–halothane contracture test (CHCT) [97] and the European *in vitro* contracture test (IVCT) [98] were born. They have proven useful in the preoperative testing of thousands of potential MHS individuals and, when performed with rigor, are considered the "gold standard" of MHS status [12]. They are, however, not perfect, with 97% sensitivity and 78% specificity for the CHCT and 99% sensitivity and 94% specificity for the IVCT [37,99–101]. Conceptually, this work proved that the development of contracture, seen in whole body studies, can be recapitulated in an isolated fiber. Clearly MHS is a malfunction of skeletal muscle alone.

3.7. Lack of histochemical findings in MH

Once it was clear that the primary cause of MH resides in skeletal muscle, it became important to determine whether any structural defects might be present that could be used in diagnosis, in understanding of the etiology of MH, or both. Thus a huge effort was invested in a search for structural abnormalities in muscle biopsies of MH patients and MHS pigs [102,103]. Initial studies were of autopsy material or of muscle biopsies from those rare survivors of an MH episode. In both cases, hypercontracted fibers were often observed. In MH survivors, individual fiber atrophy and necrosis, together with increased central nucleation, suggestive of myofiber regeneration, were also common findings [103,104]. However, abnormalities that could manifest as a pre-existing myopathy were rare. Samples showed normal fiber typing and normally structured myofibrils, sarcomeres, triads, SR and mitochondria. In some autopsy material, fibers showed dilation of the SR and swelling or rupture of mitochondria, but these occasional findings were believed to be the consequence of an MH crisis or to represent an artifact known to occur in highly irritable muscle [103–106].

In MHS pig muscle, carefully collected ante-mortem samples revealed normal muscle histology and ultrastructure, whereas post-mortem samples, collected within the first 10–12 min after immediate death by stunning, sometimes showed dramatic myofibrillar disorganization, hypercontraction and mitochondrial swelling [85]. Britt [107] suggested that the rare cases of mitochondrial swelling observed in MHS human and pig muscle samples, are caused by hypoxia, a concomitant feature of an MH episode. Eventually, it became difficult to promulgate a case for any pre-existing structural abnormality, especially when muscle biopsies of a growing number of CHCT or IVCT positive MHS patients, over a range of age groups, and especially those of MHS children, were found to be morphologically normal [13,85,102,103,106,108,109].

To put an end to the idea that pre-existing structural abnormalities occur in MHS muscle, Harrison [110] and Venable [111] made direct comparisons of porcine muscle biopsies taken immediately before, during, and just after an MH reaction. They showed repeatedly, using samples from various muscle groups, that myofibrillar organization in the muscles of MHS pigs was normal prior to an MH episode, but was dramatically disrupted within minutes after exposure to an MH-triggering anesthetic [85,110–113]. In a detailed study by Venable [111], muscle samples were collected sequentially from pigs, relaxed under non-triggering anesthesia, with a subsequent switch to graded levels of 1–3% halothane. Following halothane exposure, the morphologically normal myofibers from all muscle groups were converted into disorganized masses, with areas of intercalated super-contracted and super-stretched sarcomeres and coagulated contraction clots. This altered morphology was similar, if not identical, to that of PSE pork [85], identified occasionally on the cutting line after slaughter.

In rigorous studies on numerous MHS and MHN human muscle biopsies, Harriman presented the most convincing proof of the absence of specific pathological features in MHS human muscle prior to anesthetic exposure [102,103]. His methodical analysis of 1400 muscle biopsies from IVCT-tested MHS and control muscle samples revealed only minor, if any, differences between the samples. Seventy percent of the MHS muscle samples were seen to be normal when examined by histochemistry and electron microscopy [102,103]. He confirmed the results of earlier studies showing that the most common abnormalities in MHS muscles are slightly increased fiber size variability, an increase in the number of internal nuclei and the presence of cores in individual fibers. However, essentially the same abnormalities were observed in control samples, although with lower frequency. In a later study by Figarella-Branger et al. [114], involving 165 patients, these alternations were shown to be as frequent in control muscle samples as in those from MH patients, strongly indicating that they were non-specific. More recently, an in-depth, quantitative, histomorphological analysis of abnormalities in 132 MHS, 65 MHE and 243 MHN muscle biopsies [115] revealed similar levels of myopathologic abnormalities in muscles from all three groups. All of these studies led to the conclusion that histopathological examinations cannot aid in the diagnosis of MH and form the basis for the currently accepted view that MH is an asymptomatic myopathy.

3.8. Dantrolene as an antidote to MH

A second drug that has proven to be critical, not only to MH research, but also to MH therapy, is dantrolene sodium. In early work, Dr. Keith Ellis discovered that dantrolene is a direct acting muscle relaxant, which blocks the development of an MH episode by decreasing intracellular Ca^{2+} concentrations [116–122]. For a recent review see [25]. The discovery of the suppressant effect of dantrolene on MH reaction is recognized as one of the most important advances in MH therapy. The introduction of dantrolene into medical practice in 1979 as a clinical antidote for MH episodes dramatically decreased the mortality rate due to MH from about 80% in the 1960s to less than 10% in the1990s [25]. It is now standard practice in the Western world to maintain supplies of dantrolene in all hospital operating rooms (although not necessarily in non-hospital venues). The major disadvantages of dantrolene are its relatively short shelf life, poor water solubility, which makes rapid preparation of intravenous solutions difficult in emergency situations, and the high cost of the drug. Azumolene, an analogue of dantrolene with 30-fold greater water solubility, has been shown to be equipotent in preventing anesthetic-induced MH but, to date, it has not been introduced into practice [25]. Of interest are recent findings that dantrolene might also exert a regulatory action on the cardiac Ca^{2+} release channel, RyR2, in failing hearts [123].

Dantrolene has been shown to inhibit the RyR1 Ca^{2+} release channel directly and thus to affect the intrinsic mechanism of EC coupling [124]. It acts by reducing muscle twitch force and by shifting the sensitivity of contractile activation to higher voltages [25], but the exact mechanism of its inhibitory action is unknown. Using [^{3}H] azidodantrolene, a pharmacologically active, photoaffinity-labeled analogue of dantrolene, a dantrolene-binding site within RyR1 was identified in the region Leu590–Cys609 [125]. Dantrolene has been shown to produce a local conformational change within an MH mutant RyR1, reinforcing the idea that it alters regulatory inter-domain interactions within the Ca^{2+} release channel [126]. This finding suggests a novel mechanism for dantrolene action as a stabilizer of an inhibited conformational state of RyR1 [126].

It has been difficult to pinpoint the mechanism of dantrolene action because, even though it is clear that it diminishes Ca^{2+} release channel function *in situ*, it does not alter the activity of single RyR1 channels or of RyR1 in isolated SR vesicles of varying degrees of purity. The least complex system in which dantrolene regulates function is in HEK-293 cells expressing RyR [127]. Thus it appears that only RyR1

channels possessing a certain degree of physiological organization can be inhibited by dantrolene. This raises the possibility that dantrolene acts through an ancillary protein that regulates RyR1 function, but no such protein has been found and, moreover, dantrolene binds to RyR1. But what about structural organization within RyR1 arrays as a clue to their functional inhibition by dantrolene? Dr. Anthony Lai has analysed the way in which RyR1 tetrameric molecules are organized under physiological conditions, with subdomain 6 in each subunit interacting directly with subdomain 6 in an adjacent RyR1 tetramer [128,129]. He has proposed that each RyR1 tetramer is structurally, and probably functionally, coupled with 4 other RyR1 tetramers. Is it then possible that a group of at least 5 functionally connected RyR1 tetramers may be the minimal level of organization required for inhibition by dantrolene?

3.9. Ryanodine and the identification of the Ca^{2+} release channel

A third drug that facilitated MH research is ryanodine [130]. This plant alkaloid has the ability to regulate intracellular Ca^{2+} concentrations from a site within muscle cells rather than at the plasma membrane. Its extremely high (low nanomolar) affinity for an unknown protein inside muscle cells led to a search for its binding site. In 1985, Dr. Sidney Fleischer [131] prepared [^{3}H]ryanodine and, in a series of focused and ingenious experiments, used it in a functional test of the release of Ca^{2+} from highly purified junctional terminal cisternae. Ruthenium red blocked Ca^{2+} release, but, if the vesicles were pretreated with nanomolar ryanodine, ruthenium red could no longer prevent Ca^{2+} release. Thus ryanodine was competing, with high affinity, for a low affinity ruthenium red binding site on the Ca^{2+} release channel. In 1987, Fleischer isolated the [^{3}H]ryanodine-labeled Ca^{2+} release channel of the skeletal muscle sarcoplasmic reticulum, showed it to have a subunit size greater than 400 kDa, and named it the ryanodine receptor (RyR) [132]. Shortly thereafter, the ryanodine receptor gene, *RYR1*, would be shown to be the major genetic locus for MHS.

3.10. Discovery of luminal Ca^{2+} activation

Fleischer's studies of Ca^{2+} release from purified terminal cisternae vesicles eventually led to the discovery of a mechanism that is likely to lie at the heart of the MH reaction (see later sections). Fleischer's group showed that the ability of terminal cisternae vesicles to take up increments of added Ca^{2+} was limited [132–136]. When the size of the Ca^{2+} store reached a certain threshold, luminal Ca^{2+} triggered rapid release of the Ca^{2+} store. This process has been termed store overload-induced Ca^{2+} release (SOICR) [137,138]. Thus, in addition to Endo's high affinity cytosolic Ca^{2+} sensor site [139], there is a luminal, low affinity Ca^{2+} sensor site on the Ca^{2+} release channel [140] and each of these sensors can trigger Ca^{2+} release.

3.11. Cloning of the ryanodine receptor gene

As the technology of molecular genetics advanced during the 1980s, it became possible to clone cDNAs encoding even huge proteins. Cloning of the 15,000 bp encoding the skeletal muscle ryanodine receptor was accomplished almost simultaneously by Dr. Shosaku Numa's group for the rabbit RyR [141] and by our group for both rabbit and human RyR [142]. Shortly, thereafter, both groups cloned the homologous rabbit cardiac *RyR* cDNA [143,144] so that the protein isoform expressed in skeletal muscle was termed RyR1, encoded by the *RYR1* gene, and the protein isoform expressed in cardiac muscle was termed RyR2, encoded by the *RYR2* gene.

3.12. Linkage of MH to RYR1

Publication of the human *RYR1* cDNA sequence made it possible for geneticists worldwide to answer the question, posed two decades earlier, whether the *RYR1* gene, encoding the Ca^{2+} release channel of the skeletal muscle sarcoplasmic reticulum, is the MH locus in humans and in pigs. Back to back papers from our lab [145] and Tommie McCarthy's lab [146] in 1990 showed linkage of human MH to *RYR1* on human chromosome 19, in a region that was isologous with the location of the *HAL* gene on pig chromosome 6 that had been established earlier [147]. The next step was to find MH-causal mutations in both human and pig *RYR1*/*HAL* genes. Fujii et al. [148] reported the mutation of Arg 615 to Cys in the porcine *RYR1*/*HAL* gene and, in a follow up, established linkage of this mutation to MH in British Landrace pigs [149] with an astonishing lod score of 102 (traditionally, a lod score of 4 establishes linkage).

The discovery of the R615C mutation in pigs is one of the most successful applications of molecular genetics to an animal disease. Breeder selection for large hams and lean muscle mass inadvertently led to selection for the MH gene, possibly first in the Pietrain strain, but later, through crossbreeding and further selection, it was introduced into a variety of breeds. Haplotyping suggested that the mutation was probably the same in all of the affected breeds, making it likely that MH could be identified in the worldwide pig population by testing for the presence of the R615 mutation [148]. Indeed, this has proven to be the case, since no other pig MH mutation has been detected in the past two decades and removal of the R615C mutation from much of the world's breeding stock has dramatically reduced the incidence of PSS and PSE in the pork industry. However, because the mutation does add to muscle mass and because the Pietrain strain, in particular, has adapted to its presence, the mutation is still selected for positively by some breeders.

In parallel studies, the corresponding mutation in a human MH family (R614C) was soon identified [150] and, since then, some 300 MH-, CCD- and other congenital myopathy-associated mutations have been described in *RYR1* (Fig. 1) [17,151,152]. Because of the large and growing number of MH mutations in human *RYR1*, there is no simple genetic test for MHS in humans as there is in pigs. Thus genetic testing is still a laborious task that requires sorting through the sequence of over 15,000 bp of *RYR1* cDNA or 160,000 bp of *RYR1* genomic DNA [153]. Moreover, it is desirable that substitutions in the human *RyR1* sequence be proven to be causal disease mutations, rather than polymorphisms, by functional testing. To date, functional testing has been carried out for only a fraction of known RyR1 mutations.

RYR1 mutations have not been found in all MH families, leading to the conclusion that other MH loci must exist. Indeed, genetic linkage in some MH families have suggested an association of MH with alternate chromosomal loci [154–158], but mutations that can be considered causal have been found only in the *CACNA1S* gene (human chromosome 1q) encoding the α-1 subunit (Cav1.1) of the skeletal muscle voltage-gated Ca^{2+} channel (also referred to as the dihydropyridine receptor or DHPR) [15,18–22]. In four out of the first six non-related MH cases with mutations in Cav1.1, the affected amino acid residue was Arg1086, which was mutated either to His in three heterozygous carriers [15,18,19] or to Ser in one homozygous carrier [22]. Recently, two more Cav1.1 mutations – p.R174T [20] and p.T1354S [21] – have been associated with MH. It is easy to rationalize why mutations in Cav1.1 can cause MH, since each Cav1.1 subunit interacts physically with a single RyR1 monomer in every other RyR1 tetramer, forming a complex that is integral to the EC coupling machinery [159–162]. Functional analysis of the R1086H [163] and T1354S [21] mutations revealed that their presence in the Cav1.1 subunit enhanced RyR1 sensitivity to activation by both endogenous (voltage) and exogenous (caffeine) stimuli. Thus the effect of Cav1.1 mutations was essentially identical to that demonstrated for RyR1 MH mutations — enhanced sensitivity of the SR Ca^{2+} channel to stimuli. It was suggested that the Cav1.1 subunit functions as a negative allosteric modulator of RyR1 activation by these stimuli and that mutations in Cav1.1 may disrupt this negative allosteric influence [163].

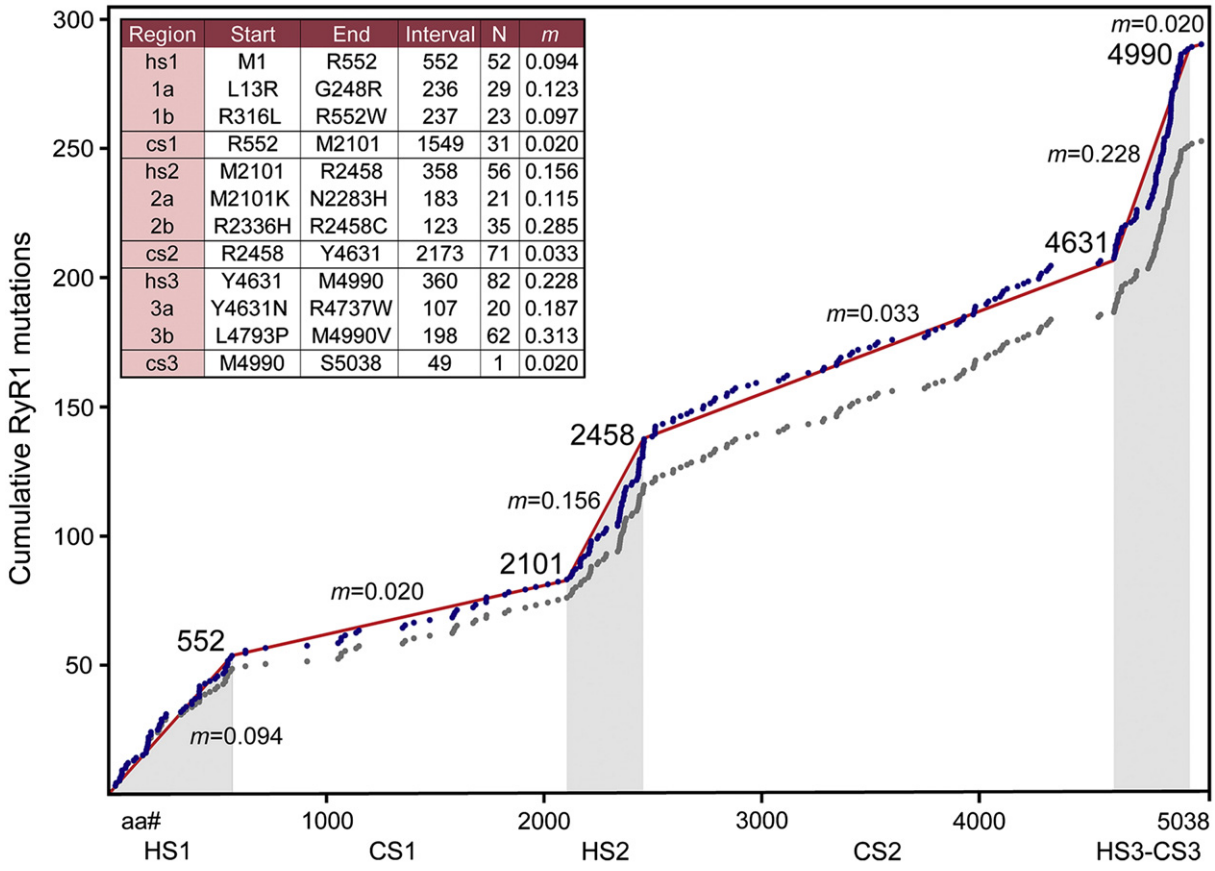

Fig. 1. A plot of the accumulation of MH- and core myopathy-associated mutations in RyR1 against amino acids 1 to 5038 in the full-length human RyR1 protein. A total of 294 RyR1 amino acids in which at least 1 mutation has been documented are plotted cumulatively as blue dots relative to their position in the amino acid sequence. In cases where redundant substitutions have occurred, they are assigned accumulation values equal to the redundancy at this site. In a second plot, using gray dots, we have assigned a value of only 1 to all mutated amino acid sites, regardless of redundancy at the site. The two plots have the same overall shape, but the rate of accumulation is lower in the gray plot. The rates at which RyR1 mutations are accumulated in relation to their location within the protein are presented graphically in the blue plot by the slopes (m) of linearized segments (red lines) representing average values across each of the different segments of the plot. Six red lines subdivide the polypeptide chain into 6 major intervals designated HS1, CS1, HS2, CS2, HS3 and CS3, where HS stands for hot spot and CS for cooler spot, each with a different slope. The slope of each interval was determined as the ratio of the number of mutations within the interval (N) divided by the length of the interval (Interval) in amino acid residues. HS1, HS2 and HS3 are regions with steep slopes, their "Start" and "End" coordinates being the points of intersection of the red lines, indicated in the graph and in the inserted table. Within each of the HS sequences there are at least 2 subsequences, designated "a" and "b", in which the slopes differ from the average, some being much steeper. The intervals of these subsections are delineated in the inserted table by the first and the last mutated amino acid residue within the interval.

Recently, MH-like episodes, reversed or prevented by dantrolene, have been shown to occur in mice with a knockout of the *Casq1* gene [164] encoding skeletal muscle calsequestrin, a low affinity, high capacity, Ca^{2+} binding/buffering protein located in the SR lumen [165,166]. Later, we will return to the rationale for why the loss of calsequestrin function might cause MH.

3.13. Linkage of CCD to RYR1

Because of the clinical association of some cases of MH with CCD, the next step was to link CCD to mutations in the *RYR1* gene. Again, our lab [167] and Tommie McCarthy's lab [38] led the way in demonstrating linkage in a number of CCD families. To date, no other candidate gene for CCD has been proposed. While CCD mutations have been found throughout the *RYR1* gene, it is now clear that the majority of CCD mutations are concentrated in the C-terminal end of RyR1 where 6 transmembrane (TM) sequences in each of the 4 subunits assemble to form the ion pore region of the ion channel [168,169].

3.14. Distribution of MH and CCD mutations within RYR1

After the first MH mutation was found at amino acid 615, a number of mutations were located near the N-terminus of RyR1. In a second wave of discovery, MH and CCD mutations were found in a central region between amino acids 2100 and 2500 and, in a third wave, mutations were found in the C-terminal region surrounding the channel-forming region. These early studies suggested that there are three mutation hot spots in *RYR1*, designated MHS1, MHS2 and MHS3 [157]. However, with time, some 300 mutations causing RyR1-related disorders have been found throughout virtually the entire length of RyR1 [17,151].

In Fig. 1, we have plotted the rate of accumulation of those amino acid sites at which MH- or core myopathy-related mutations occur (ordinate) against the 5038 amino acids that comprise human RyR1 (abscissa). At several sites, multiple substitutions associated with MH or core myopathy have occurred (redundant mutations). To capture this redundancy of causal mutations, we have assigned accumulation values equal to the redundancy at each site in the plot created with blue dots. In a second plot, using gray dots, we assigned a value of 1 to all mutated amino acid sites, regardless of redundancy. The two plots have the same overall shape, but a different accumulation rate.

Presentation of the accumulation of mutations in these ways permits a redefinition of the boundaries of the 3 hot spots. The three mutation hotspots are now identified as HS1, HS2 and HS3 and intervening regions are designated as CS1, CS2 and CS3 where HS stands for Hot Spot, since the regions include mutations for both MH and core myopathies, and CS stands for Cooler Spots, with a lower density of cumulative mutations. The plots clearly show that, with a

few exceptions such as the mutant-free interval between amino acids 4338 and 4558, mutations are scattered throughout most of the RyR1 protein.

Different slopes in the red lines defining the averaged rates of accumulation of mutations in the blue plot indicate different regions of the molecule in which mutations are accumulated with higher and lower density. The HS1 region lies between amino acids 1–522 (m = 0.094); the celebrated R615C mutation may not lie within hot spot 1! HS2 is comprised of amino acids 2101–2548 (m = 0.156) and HS3 is comprised of amino acids 4631–4990 (m = 0.228). Predicted transmembrane (TM) sequence 1 (4557–4576) [168] is excluded from HS3, but TM sequences 2 (4637–4662), 3 (4776–4800), 4 (4803–4825), 5 (4834–4854) and 6 (4911–4935) and the ion selectivity filter (4896–4899) clearly lie within HS3. Within the HS regions there are sub-regions in which the rate of accumulation of mutations is higher than the average rate across the entire HS region. Region HS3b, with a slope of 0.313 (~1 mutation in every 3 amino acids!), encompasses amino acids 4793–4990, which excludes TM sequence 2 but includes TM sequences 3, 4, 5 and 6 and the ion-selectivity sequence lying at the heart of the ion pore. HS2b, with a slope of 0.285 encompasses amino acids 2336–2548.

Comparison of the blue and gray plots shows that they have the same overall shape, but a different accumulation rate. The value of this comparison lies in its definition of where redundancy occurs. Thus the spacing between the blue and gray lines runs more or less parallel throughout regions CS1 and CS2, but the width of the blue-gray spacing is increased dramatically after HS1, more after HS2 and even more after HS3, indicative of the fact that multiple mutations of the same amino acid are more concentrated in the 3 HS regions than elsewhere in the molecule.

A comparison of the full sequences of all known RyR isoforms shows 3 regions of high sequence diversity. They have been designated divergent regions 1, 2 and 3 (D1, D2 and D3) [170–172]. D1, consisting of amino acids 4254–4631, contains the mutant-free interval (4338–4558) referred to above. The entire D1 region in human RyR1 contains only 9 mutations, including that in position 4631; the human D2 region, amino acids 1342–1403, contains 3 mutations, including that in position 1342; and the human D3 region, amino acids 1872–1923, contains 1 mutation. Thus, the relative lack of functional mutations in these regions is the mirror image of the lack of conservation of these RyR1 sequences.

Dr. Noriaki Ikemoto and his colleagues have provided a possible explanation for mutation clustering in HS1 and HS2 by proposing that interactions between the N-terminal and central domains of RyR1 are involved in regulation of the Ca^{2+} release channel, with these interacting domains forming a 'domain switch' [173,174]. According to their hypothesis, in the resting or non-activated state, the N-terminal and central domains make close contact at several sub-domains (domain zipping). The conformational constraints imparted by the "zipped" configuration of these two domains stabilize and maintain the closed state of the Ca^{2+} channel. Stimulation *via* EC coupling or pharmacological agents weakens these critical inter-domain contacts, resulting in a reduction of conformational constraints (domain unzipping), thereby lowering the energy barrier for Ca^{2+} channel opening. Weakening of the inter-domain interactions may also occur *via* mutations of critical residues within these regions.

The fact that mutations are spread across the entire length of RyR1 raises the question of how can so many mutations impinge on the same function — here proposed to be spontaneous Ca^{2+} release? Two points can be made: first, RyR1 mutations are identified because they all cause MH or core myopathies — they really do have an effect on RyR1 function! Second, in our earlier studies of mutations in the Ca^{2+} pump, we were able to define mutations that had specific effects [175–178]. For example, we identified all of the key residues in the TM sequences that bind Ca^{2+} and, with others, identified the residues in two cytosolic domains that bind ATP. However, in one group of mutations, amino acids scattered throughout all domains of the molecule all blocked a critical conformational transition between E1P and E2P. We called these "conformational change" mutations [176]. When Dr. Chikashi Toyoshima determined the crystal structures of nine conformational states of SERCA1 [179,180] it was apparent that there were huge movements of all of the 3 cytosolic domains and several of the 10 TM helices. The A domain rotated 110°, and some of the TM helices moved in and out of the membrane (http://www.nature.com/nature/journal/v432/n7015/extref/nature02981-s6.mov). Evidence now emerging from low resolution structures of RyR1 [181], strongly suggests that when high resolution structures of different conformations of RyR1 are obtained, we will see that long-range conformational changes utilize most domains within the molecule, thereby explaining how wide-spread mutations that affect conformational changes can impinge on a single function: increasing the probability of spontaneous Ca^{2+} release. Indeed, such long-range conformational changes lie at the heart of the postulate that inter-domain unzipping of RyR1 subunits is involved in the activation of Ca^{2+} release channels [174].

3.15. Functional analysis of MH and CCD mutant proteins

Cloning of full-length forms of RyR1 opened the door to functional analysis of mutant proteins by expressing them in a non-homologous cell system such as HEK-293 cells that do not express endogenous RyR1. Following transfection, RyR1 mutants are expressed in the ER, an organelle that functions like the SR to store Ca^{2+} and regulate Ca^{2+} signaling in non-muscle cells. Ca^{2+} release transients can be measured readily in these cells using Fura2 to image cytosolic Ca^{2+}. Cell permeable agonists such as caffeine and halothane can then be administered to create dose response curves for induced Ca^{2+} release.

The first experiments, conducted by Dr. Jeifei Tong [182–184], recapitulated and extended what had already been demonstrated by the addition of caffeine and halothane to intact skeletal muscle fibers. The RyR1 proteins carrying MH mutations were all activated by both caffeine and halothane at concentrations lower than those that stimulated Ca^{2+} release with normal channels — they were hypersensitive to these agonists. It was also possible to measure resting cytosolic Ca^{2+} concentrations in the presence of expressed RyR1 proteins and, since the amplitude of the maximal Ca^{2+} release response is proportional to the size of the luminal Ca^{2+} store, it was possible to measure the size of Ca^{2+} stores [184]. A correlation was found between elevated cytosolic Ca^{2+} and a decrease in the level of the Ca^{2+} store. When this was extended to CCD mutant forms of RyR1, the resting cytosolic Ca^{2+} was even more elevated than with MH mutants and the size of the Ca^{2+} store was correspondingly diminished [182–184]. All of these experiments, carried out in a model system, provided proof that: (1) MH mutant Ca^{2+} release channel are hypersensitive to both caffeine and halothane; and (2) in the presence of caffeine or halothane, they can cause a dramatic shift in cellular Ca^{2+} regulation by increasing resting cytosolic Ca^{2+} and diminishing Ca^{2+} stores. The increase in cytosolic Ca^{2+} is consistent with the ability of these channels to promote contracture in muscle cells and the decrease in luminal Ca^{2+} is consistent with the idea, to be developed in a later section, that MH arises from mutant channels that, in the presence of halothane, have a lowered threshold for activation by luminal Ca^{2+}.

3.16. The discovery of "EC-uncoupled" CCD RyR1 mutants

In early studies, when MH and CCD mutants had been located only in cytosolic domains of RyR1, a mutation lying in the center of the proposed RyR1 selectivity filter (Ile4898 to Thr) [169,185] and causing a severe form of CCD [183] was in uncharted territory. Studies of this RyR1 mutant in HEK-293 cells showed that the mutant channel exhibited little or no caffeine-induced Ca^{2+} release [183]. This was consistent with either a loss of Ca^{2+} release channel function or extreme depletion of the Ca^{2+}

store by excessive spontaneous activation of the channel. Measurement showed that cytosolic Ca^{2+} was high, thus favoring a very leaky channel, but this conclusion turned out to be wrong. Dr. Robert Dirksen had developed a more physiological system for measuring Ca^{2+} release by transfecting myotubes from dyspedic (*Ryr1*-null) mice with rabbit *RYR1* mutant cDNAs, thereby reconstituting EC coupling in these myotubes. His studies showed that the I4898T mutant was nonfunctional with respect to orthograde Ca^{2+} release, but retained the retrograde function of stimulating Ca^{2+} entry through normal Cav1.1 [186,187]. These findings have been confirmed in functional studies on skeletal myotubes from an I4895T mutant knockin mouse line [188].

These data on functional effects of CCD mutations presented a paradox, since CCD and the formation of cores could be caused by two types of mutants, those that were excessively active, elevating cytosolic Ca^{2+} levels, and those that had diminished capacity for Ca^{2+} release and did not elevate cytosolic Ca^{2+} levels [189–191]. In later sections, we will describe findings with the I4898T and Y522S mouse lines that will help to explain how core lesions form and how they might be produced by functionally different RyR1 mutations.

3.17. Generation of MH and CCD mouse lines

With a growing number of MH and CCD-associated RyR1 mutations, the need arose for the generation and comparative study of animal models carrying different mutations. Recently 3 mouse lines carrying knockin *Ryr1* mutations have been generated. A line carrying the $Ryr1^{R165C/+}$ mutation was developed by Dr. Paul Allen [192] and a line carrying the $Ryr1^{Y524S/+}$ mutations was developed by Dr. Susan Hamilton [193]. These mutations are analogous to the heterozygous human mutations $RYR1^{R163C/+}$ and $RYR1^{Y522S/+}$, which have been described as being causal of MH [17]. *In vitro* and *in vivo* functional studies of these mutations showed that both mutations render the RyR1 channel highly sensitive to activation and lead to the elevation of cytosolic Ca^{2+} levels. The third knockin line carries the EC-uncoupling mutation, $Ryr1^{I4895T/+}$ [177], analogous to the human mutation, $RYR1^{I4898T/+}$, one of the most frequent and highly penetrant CCD mutations in humans [17,183,194]. It causes a severe form of CCD with muscle weakness, but no apparent susceptibility to MH.

R163C has been found in more than 20 MH cases worldwide [17,195–197]. In different MH families, the mutation shows a variable clinical phenotype, manifesting as either MH only, as MH with cores, or as MH/CCD. Y522S has been found in two MH families [38,198]. In both families there were no clinical signs of muscle weakness. In the family described by Quane et al. [38], two MHS family members had cores in their skeletal muscle biopsies. In this case, Y522S was presented as an MH mutation "with associated central cores" [38]. This was one of the earliest attempts to discriminate between MH mutations presenting with cores, but without muscle weakness, and MH/CCD mutations, presenting with both cores and muscle weakness.

Heterozygous mice of both R163C (RC/+) and Y522S (YS/+) lines develop fulminant MH episodes when exposed to volatile anesthetics or following short-term heat exposure [192,193]. Studies of these mouse lines have permitted the acquisition of detailed insight into the etiology of MH induced by N-terminal leaky MH-causative RyR1 mutations [199–202]. The Y522S line has also served as a valuable tool for the elucidation of core formation caused by an MH-associated RyR1 mutation [203].

Heterozygous mice of the I4895T (IT/+) line [204], carrying an uncoupling C-terminal RyR1 mutation, exhibit a slowly progressive myopathy with skeletal muscle weakness and age-dependent formation of cores in their skeletal muscle, strikingly similar to the pathology of RyR1-related core myopathies [29]. The IT/+ mouse line holds great promise for investigation of the etiology and pathogenesis of not only CCD, but also of the entire group of congenital muscle weakness disorders, including multiminicore disease and nemaline rod myopathy, since progressive formation of minicores, cores and rods has been found in the skeletal muscle of I4895T mice [204].

3.18. Insights into the mechanism of core formation in CCD and MH mouse models

Examination of skeletal muscle samples from IT/+ mice [204] has provided insights into the structural changes leading to core formation that are caused by an EC-uncoupled CCD mutation (Fig. 2). The earliest signs of ultrastructural abnormalities, in 6-week-old mice, consist of irregularities of myofibrillar organization, focal myofibrillar thinning, misalignment, and intermingling. This pattern suggests that local abnormalities in myofibrillogenesis occur sporadically. Analyses of muscle samples from older mice revealed that these early structural abnormalities undergo a transition from small, compacted areas resembling minicores in younger animals to more extensive cores in mice 6 months of age. By 12–18 months, nemaline rods are observed in extended cores. Mitochondria are progressively displaced from the center of the cores and accumulate at the core periphery, while retaining their normal non-swollen structure.

There is suggestive evidence for a similar age-dependent transition in myofiber lesions in the skeletal muscles of CCD patients. In a few CCD cases where repetitive skeletal muscle sampling was possible, no abnormalities or only minicores were found in muscle biopsies from younger patients, whereas cores were observed in muscle biopsies taken at a later age [205,206] or in muscle biopsies of affected parents [207–209]. Nemaline rods have also been detected in some CCD cases [47,48,205,210].

Accordingly, it had been proposed that there is a common origin for minicore, core, and rod lesions [205,210], but it had not been possible to determine whether CCD cases with a mixed presentation of lesions represent a typical course of disease progression or reveal individual variability of the disease manifestation. The fact that IT/+ mice, express a full spectrum of minicores, cores and rods, in an age-dependent progression, provides independent recent evidence supporting the earlier proposals that these structural abnormalities represent consecutive stages of skeletal muscle disease progression.

The relevance of the progressive pathological changes observed in the I48985T mouse model to the disease progression in human CCD cases has been demonstrated in a recent study in which rods were found in the skeletal muscle samples of infant twins affected by an

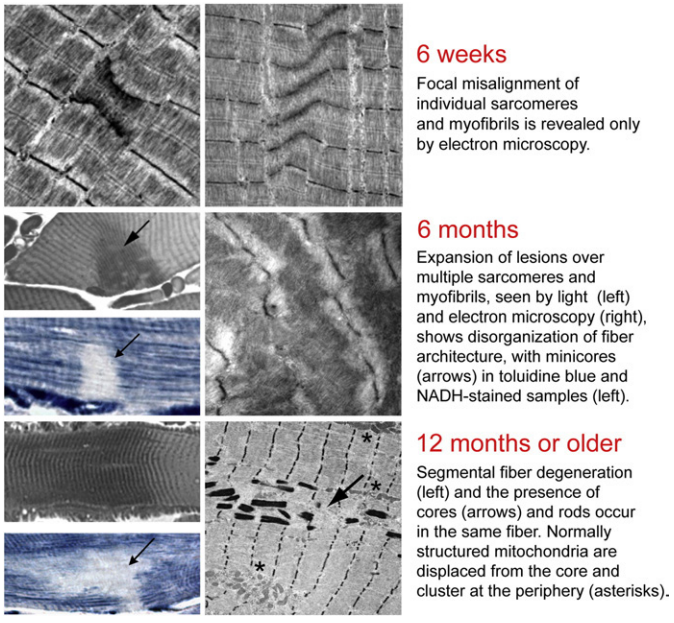

Fig. 2. Age-dependent accumulation of lesions within the skeletal muscle fibers of I4895T/+ mice.

extremely severe and lethal CCD, that was associated with the *de novo* mutation of Ile4898 to Thr [194]. Taken together, these data from the I48985T CCD mouse model and I4898T human carriers show that structural abnormalities, conventionally attributed to distinct congenital myopathies and classified as MmD, CCD, and myopathy with rods, do have a common etiology and pathogenesis [205,210].

Age-dependent structural abnormalities have also been found in muscle fibers from the YS/+ mouse model carrying a knockin of the heterozygous leaky channel mutation, Y522S [199,203]. YS/+ mice develop localized regions of mitochondrial and sarcotubular disruption as early as 2 months of age. In these focal regions of disruption, mitochondria are swollen, presenting a more translucent matrix, and exhibit significant cristae remodelling. At this early stage, changes in organelle structure are not accompanied by myofibrillar contracture, suggesting that they do not result from focally elevated Ca^{2+}. However, contracture and mitochondrial swelling resulting from spontaneous Ca^{2+} release might follow different time courses so that they would not have to appear together in the same fixed section. A contracture might be reversible and disappear in a very short time frame, whereas the irreversibly swollen corpse of a mitochondrion, disrupted during an earlier episode of spontaneous Ca^{2+} release, might linger for a much longer period as the only remaining evidence of that earlier episode. Between 3 and 12 months, the regions exhibiting contracted myofibrils that lack SR and mitochondria, become prevalent and increase in area. By 12 months, these regions encompass a larger area, they lack contractile elements, SR, and mitochondria and they resemble unstructured cores in human skeletal muscle biopsies [199,203].

The disruption of about 9% of mitochondria in young YS/+ mice raises the question of how this structural feature relates to long-term core formation. There are at least two possibilities; one is that disruption of mitochondria, with its attendant potential for triggering apoptosis, leads to the destruction and replacement of individual muscle fibers. To date, there is no evidence that this occurs. The other is that these focal areas develop repeatedly, but do not progress further and may even be reversible, so that damage to the myofiber is surmountable. At some point, however, the damage becomes irreversible so that the contraction cores progress to the larger unstructured cores, as documented in older YS/+ mice. Overall, these studies of the ultrastructure of CCD and MH mouse models suggest that EC-uncoupled IT/+ mutations and leaky YS/+ mutations result in two distinct, progressive pathologies.

3.19. Characteristics of RyR2 mutations in CPVT

To date, only two genes have been definitively associated with MH in humans: *RYR1* and *CACNA1S* [158], but *Casq1*-null mice, developed by Dr. Feliciano Protasi, have been shown to respond to halothane with a typical MH response that is reversed by dantrolene [164,211]. This finding highlights the analogy between MH and CPVT, an arrhythmogenic cardiac disorder caused by mutations in both *RYR2* and *CASQ2* genes, the isoforms expressed in the heart [137,212–216] and raises the point that similarities between MH and CPVT can help us to understand the underlying causes of these two diseases.

It is generally accepted that CPVT arrhythmias result from repetitive, premature activation of RyR2, in other words, spontaneous Ca^{2+} release. As described above, RyRs are activated by the elevation of luminal Ca^{2+} beyond a threshold at which SOICR is triggered [133,214,217–219]. We have also discussed above how RyR function can be measured following its expression in HEK-293 cells. In a remarkable series of experiments, Dr. Wayne Chen's group adapted this system to measure the effect of luminal Ca^{2+} on spontaneous Ca^{2+} release by wt RyR2 expressed in HEK-293 cells [127,137,138,220,221]. They elevated luminal Ca^{2+} by incremental elevation of extracellular Ca^{2+}, which, in turn, elevates cytosolic Ca^{2+} through plasma membrane leaks and then elevates the luminal Ca^{2+} store through the action of sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) pumps. They found that the percentage of cells in which spontaneous oscillations of Ca^{2+} release and reuptake occur was dramatically increased as extracellular Ca^{2+} was elevated incrementally. At each incremental stage of elevation of extracellular Ca^{2+}, it was possible to measure the size of the luminal Ca^{2+} store. It doubled in size, but reached a maximum with about the same increment of external Ca^{2+} that maximally activated spontaneous Ca^{2+} oscillations. Thus there was a clear relationship between the elevation of luminal Ca^{2+} and the frequency of Ca^{2+} oscillations. As a further twist, the addition of 0.3 mM caffeine, which was too low to activate the Ca^{2+} release channel by itself, increased the frequency of Ca^{2+} oscillations dramatically. These observations confirm, first, that wt RyR2 has an intrinsic threshold at which luminal Ca^{2+} can activate spontaneous Ca^{2+} release, and second, that caffeine has the ability to lower the threshold at which luminal Ca^{2+} can activate spontaneous Ca^{2+} release in cells transfected with wt RyR2.

The next step was to measure the effect of elevating luminal Ca^{2+} on the spontaneous oscillations of CPVT mutations. RyR2 CPVT mutant channels, expressed in HEK-293 cells, were activated by much lower luminal Ca^{2+} levels than wt [137,138]. In fact, the level of activation was equivalent to that seen in wt cells supplemented with 0.3 mM caffeine. Critically, the size of the Ca^{2+} store for all CPVT mutants studied was *decreased* as the frequency of Ca^{2+} oscillations was increased. This implies that the CPVT mutation had lowered the intrinsic threshold at which luminal Ca^{2+} can activate spontaneous Ca^{2+} release. When single channel activities of RyR2 CPVT mutants were measured in planar lipid bilayers with 45 nM cytoplasmic Ca^{2+}, incremental elevation of luminal Ca^{2+} from 0 to 1000 μM increased the probability of opening (P_o) to 0.3–0.4 for CPVT mutant RyR2s *vs.* 0.02 for wt RyR2. These results demonstrate that RyR2 CPVT mutant channels, in isolation from other SR proteins, manifest a lower SOICR threshold than wt RyR2, as illustrated in Fig. 3.

3.20. Characteristics of RyR1 mutants in MH

What happens when an experiment analogous to that done with RyR2 CPVT is carried out with MH mutations in RyR1? In a comparable study, spontaneous Ca^{2+} release was studied for RyR1 R615C MH mutant Ca^{2+} release channels expressed in HEK-293 cells [127,220]. The findings were consistent with the findings for RyR2 CPVT mutations. Wt RyR1, expressed in HEK-293 cells, showed an increased rate of spontaneous Ca^{2+} release as extracellular Ca^{2+} was elevated above physiological levels in the presence of 0.7 mM halothane, a concentration of halothane that does not stimulate spontaneous Ca^{2+} release from wt RyR1 in the presence of physiological levels of extracellular Ca^{2+}. A combination of elevated luminal Ca^{2+} and halothane increased spontaneous Ca^{2+} release in both wt and MH mutant RyR1, but the rate and frequency of spontaneous Ca^{2+} release was increased 20 fold by 0.7 mM halothane in MH mutant RyR1 [127]. In another experiment in which the transfected R615C RyR1 MH mutant channel was induced to undergo rapid spontaneous Ca^{2+} oscillations in the presence of elevated Ca^{2+} and 2 mM caffeine, the addition of 100 nM dantrolene blocked Ca^{2+} oscillations reversibly, apparently by suppressing SOICR in these transfected HEK-293 cells. These results demonstrate that RyR1 MH mutations lower the threshold for luminal Ca^{2+} activation, that volatile anesthetics and caffeine further lower the SOICR threshold, and that dantrolene suppresses SOICR, as illustrated in Fig. 3.

The conclusions from these recent studies are fully in line with conclusions reached in earlier studies performed with samples from MH pigs. In a series of experiments, Nelson et al. [219,222,223] showed that the MH defect in pig muscle lies in Ca^{2+} loading-dependent activation of Ca^{2+} release from the SR. Although he called the process Ca^{2+}-induced Ca^{2+} release, it is clear that he was actually referring to what is now termed store overload-induced Ca^{2+} release, or SOICR.

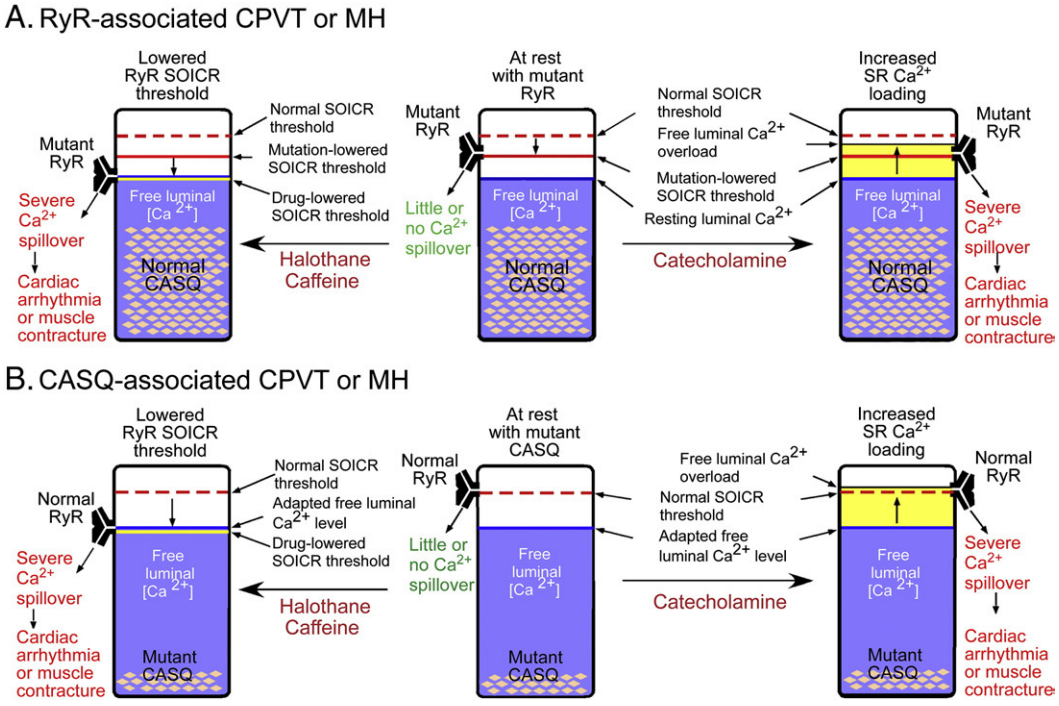

Fig. 3. A model unifying the etiology of CPVT and MH arising from mutations in *RYR* or *CASQ* genes. The different thresholds for SOICR and the free SR luminal Ca^{2+} levels in CPVT or MH, associated with mutations in *RYR* (A) or *CASQ* genes (B), are illustrated in the resting state (*middle panels*) and stimulated states (*left and right panels*). The normal SOICR threshold is depicted as a dashed red bar. The SOICR threshold, that is lowered as a consequence of RyR mutations, is depicted as a solid red bar. The SOICR threshold that is lowered by drugs (halothane or caffeine) is depicted as a solid yellow bar in the *left* panels. The SR free luminal Ca^{2+} level is represented as a blue area. The pink lozenges within the blue areas represent Ca^{2+}–Casq complexes, which are reduced as a result of point or truncation mutants (as depicted) or abolished as a result of null mutations (not depicted). The yellow areas above the blue areas in the *right* panels represent an elevation, even if only transient, in the free Ca^{2+} levels that we propose will occur when SERCA activity is enhanced by catecholamines. When the SOICR threshold is lowered below the SR free luminal Ca^{2+} level (*left panels*), or when the SR free luminal Ca^{2+} level surpasses, even transiently, the normal SOICR threshold (*right panels*), SOICR occurs, leading to a spillover of SR Ca^{2+} that can trigger spontaneous Ca^{2+} release, cardiac arrhythmias or skeletal muscle contracture (reprinted from [140]).

3.21. Characteristics of CASQ mutants in CPVT and MH

In developing the argument that MH and CPVT share a common feature – spontaneous Ca^{2+} oscillations triggered by SOICR – studies of the effect of calsequestrin deficiency play a prominent role. Calsequestrin increases the total SR luminal Ca^{2+} content through its highly cooperative, high capacity, low affinity Ca^{2+} binding that is realized through the formation of Ca^{2+}–Casq filaments that are built through front-to-front and back-to-back polymerization, accomplished through arm exchange and domain swapping [165,224]. As folding progresses, cooperative interactions bring acidic surface residues together, literally creating new Ca^{2+} binding sites. Thus Casq acts as a buffer against abrupt elevation of the free Ca^{2+} levels in the SR lumen as Ca^{2+} is pumped inwards by the action of SERCA pumps. CPVT mutations such as R33Q affect arm exchange and domain swapping that are critical to proper folding, whereas other mutations such as LI67H affect the folding of the hydrophobic core; in either case, highly cooperative, high capacity Ca^{2+} binding is disrupted [224].

The Ca^{2+}–Casq filaments are connected to the luminal side of RyR through triadin (Trd), a TM protein that binds to RyR1 through intramembrane interactions and to filamentous Casq through luminal interactions [225,226]. This arrangement of proteins situates Ca^{2+}–calsequestrin as a filamentous "wire" that can conduct Ca^{2+} directly into the RyR pore [165,166,227–229]. Ablation of Casq by mutations in *CASQ1* or *2* will reduce the total Ca^{2+} binding and buffering capacity of the lumen by the amount that can be ascribed to Casq. Complete ablation of Casq occurs in *Casq*-null mice, but point or truncation mutations in Casq that affect key residues involved in arm exchange and domain swapping or hydrophobic core stability can disrupt the ability of mutant Casq to polymerize front-to-front and back-to-back, thereby abolishing cooperative, high capacity Ca^{2+} binding [224,230]. In the absence of Casq or in the presence of Casq with impaired polymerization [230], and especially in a state where SERCA is fully activated by adrenergic stimulation, diminished luminal Ca^{2+} buffering will permit luminal Ca^{2+} concentrations to overshoot the *normal threshold of wt RyR* for SOICR, causing either an arrhythmia or an MH episode, as illustrated in Fig. 3.

While it is generally agreed that the spontaneous release of SR Ca^{2+} is the primary process associated with the development of MH, there is controversy about whether spontaneous Ca^{2+} release is a property intrinsic to RyR or whether Casq linked to RyR through interactions with Trd is involved in triggering spontaneous Ca^{2+} release. In another publication [140], we have listed the reasons why we do not think that Casq is a direct regulator of RyR.

4. A proposal that SOICR is the common triggering mechanism for CPVT and MH episodes arising from mutations in *RYR* and *CASQ* genes

As a summary of the observations on MH and CPVT discussed to this point, CPVT and MHS individuals do not display abnormalities in muscle structure or function at rest. Both result from abnormal, repetitive Ca^{2+} cycling, characterized by spontaneous Ca^{2+} release and reuptake. An arrhythmogenic CPVT episode is triggered in response to physical or emotional stress, whereas a fulminant MH episode, characterized by skeletal muscle contracture, hypermetabolism and fever is triggered in response to volatile anesthetics, stress or elevated temperature. Causal *RYR* mutations are dominant, reflecting the dominant impact of the mutations on channel function, whereas causal *CASQ* mutations are recessive, reflecting the fact that it is the dramatic reduction in cooperative, high capacity, low affinity

luminal Ca^{2+} binding that is critical to the induction of an MH episode or arrhythmia.

We believe that a common mechanism underlies the triggering of arrhythmias caused by *RYR2* or *CASQ2* CPVT mutations and the muscle contracture caused by *RYR1* or *CASQ1* MH mutations (Fig. 3). We propose that, in all cases, the free SR luminal Ca^{2+} level repetitively exceeds the threshold for SOICR, leading to spontaneous Ca^{2+} release. In CPVT, RyR2 mutants have a lower than wt threshold for SOICR. Under conditions of exercise or stress, when the β-adrenergic pathway is stimulated, phospholamban (PLN) becomes phosphorylated by PKA. PLN is a critical regulator of cardiac contractility [231], which suppresses Ca^{2+} uptake into the SR by reducing the apparent affinity of SERCA2a for Ca^{2+}. When phosphorylated, the inhibitory function of PLN is lost and SERCA becomes fully active, bringing about a surge in Ca^{2+} entry into the lumen of the SR. This surge causes the level of free Ca^{2+} to overshoot the SOICR threshold of mutant RyR2, which has been *lowered* by the CPVT mutation. This sets up repetitive oscillations consisting of an increased rate of Ca^{2+} entry into the SR, together with an increased rate of spontaneous Ca^{2+} release that is triggered by a much lower level of luminal Ca^{2+}. Although the amplitude of Ca^{2+} release is lowered in proportion to the lowered size of the Ca^{2+} store, the general elevation of cytosolic Ca^{2+} is sufficient to increase the rate of Na^{+}/Ca^{2+} exchange, which depolarizes the membrane sufficiently to set up delayed after depolarizations (DADs) that manifest as a triggered arrhythmia. Casq2 mutants, which fail to perform their function as luminal Ca^{2+} binding and buffering proteins, will no longer be able to provide the buffering capacity required to prevent free luminal Ca^{2+} levels from overshooting the *normal* RyR2 SOICR threshold when SERCA-dependent Ca^{2+} uptake is increased. In either case, repetitive SOICR manifests as triggered arrhythmia.

In MH, RyR1 mutants have a lowered threshold for SOICR. In the presence of halothane, which *further lowers its mutation-lowered* SOICR threshold, RyR1 will be triggered to open when the free SR luminal Ca^{2+} level overshoots its *lowered* SOICR threshold. Casq1 mutants have a reduced luminal Ca^{2+} binding and buffering capacity, allowing free luminal Ca^{2+} levels to overshoot the *halothane-lowered* SOICR threshold of wt RyR1. In either case, a cycle is initiated in which SERCA pumps only partially refill the luminal Ca^{2+} store before SOICR is triggered. Repetitive SOICR elevates cytosolic Ca^{2+} to provide signals for skeletal muscle contracture and hypermetabolism, whereas fever arises from enhanced ATP hydrolysis by SERCA and myosin ATPase [140].

While we propose that SOICR is the underlying cause of these disorders, the overall process of spontaneous Ca^{2+} release almost certainly incorporates elements of other mechanisms that have been described: inter-domain unzipping of RyR subunits, changes in RyR redox status, increased rate of Ca^{2+} entry into the SR lumen following adrenergic stimulation resulting from emotional or physical stress, changes in RyR phosphorylation status, and elevated enzymatic function arising from elevated temperatures.

5. A proposal that non-uniformity of Ca^{2+} Release Unit (CRU) function underlies phenotypic variability, variable penetrance and core formation in CCD and RyR1-related congenital myopathies

As an introduction to this final section, we restate that *RYR1* is the only known causal gene for CCD and related core myopathies. RyR1 is a tetramer of 2.3 MDa, assembled from identical ~565 kDa subunits. In the junctional terminal cisternae of the SR, tetrameric RyR1 molecules form ordered 2D arrays, in which each of the four subunits in every other RyR1 tetramer is physically coupled to a DHPR complex located in the transverse tubular membrane [159–162], and each RyR1 tetramer is physically coupled to 4 other RyR1 tetramers through interactions centered around subdomain 6 [128,129]. The precise organization of these two systems, referred to as Ca^{2+} release Units or CRUs, is a critical feature of skeletal muscle EC coupling, since cooperative functional interaction within this unit accounts for the temporal and spatial uniformity of Ca^{2+} release that underlies the uniformity of skeletal muscle contraction.

The abrupt transition from sarcomeric register to lack of register was one of the most prominent ultrastructural abnormalities in CCD muscle in the earliest publications on human CCD [35,69]. A plausible explanation for the lack of sarcomeric register is a lack of uniformity of Ca^{2+} release, arising from a lack of functional uniformity among the CRUs that regulate the contraction of those sarcomeres that lie within the CRU regulatory domain. An important question is how could non-uniformity of Ca^{2+} release arise from a heterozygous Ca^{2+} release channel? The answer may lie in the way in which the tetrameric channel is assembled.

CCD is typically associated with heterozygous missense RyR1 mutations, implying that expression of both wt and mutant RyR1 subunits occurs within the skeletal muscle. Random combination of subunits into RyR1 tetramers would produce six possible variants: homotetrameric wild type channels of normal activity; homotetrameric mutant channels of compromised or null activity; and heterotetrameric channels of intermediate activity arising from four possible tetrameric arrangements of wt:mutant subunits (3:1; 1:3; 2:2, side by side; 2:2, diagonally apposed). The 16 possible arrangements of subunits into tetramers are illustrated in Fig. 4. The level of expression of mutant subunits may vary, depending on the nature of each individual mutation. However, if we assume that the expression of wt and mutant subunits is similar, then 1/16 of all RyR1 tetramers would be fully active wt homotetramers; 1/16 would be mutant homotetramers of maximally compromised activity; and 14/16 would exhibit an intermediate level of functional competence, which would likely be unique for each mutation [231,232]. The random association of two different subunits within each tetramer, followed by the

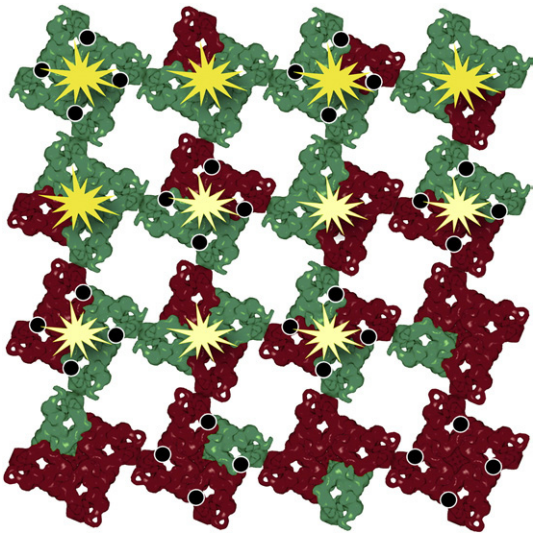

Fig. 4. RyR1 tetramer grid showing all sixteen combinations of subunit assembly from equal numbers of wt (green) and mutant (red) subunits and the resulting relative function of the different channels. Superimposed on the grid are black dots representing DHPR tetramers. The relative amplitude of Ca^{2+} release from each tetramer is depicted by a yellow "puff", approximating the relative results of single channel recordings for heterozygous I4898T mutations in which 3 groups were observed: Dark yellow puff; normal (100%) Ca^{2+} conductance (5 channels out of 20); Light yellow puff; decreased (88%) Ca^{2+} conductance (6 out of 20); and no puff, corresponding to 0 Ca^{2+} conductance (9 out of 20). The presence of 3 functionally different groups of RyR1 tetramers in a roughly equal ratio is consistent with the view that the group with normal channel activity includes not only wt homotetramers, but also tetramers with 3 wt and 1 mutant subunits, for a cumulative fraction of 5/16; that the group of channels with decreased Ca^{2+} channel activity is formed by tetramers with 2 mutant and 2 wt subunits, for a cumulative fraction of 6/16; and that the group of inactive channels includes not only mutant homotetramers, but also tetramers with 3 mutant and 1 wt subunits, for a cumulative fraction of 5/16 [236].

random association of 16 different types of tetramers within each CRU, would now set the stage for *non-uniformity* of Ca^{2+} release among CRUs. This would then lead to *non-uniformity* of contraction among sarcomeric domains, each of which could be regulated by a functionally different CRU. The effects of non uniformity of Ca^{2+} release could be amplified beyond the level of the sarcomere; to the myofibril the myofiber and the muscle fasciculus. It could also alter other Ca^{2+}-dependent processes within the skeletal muscle, such as energy production, myofibrillar structure and maintenance, thereby contributing significantly to the phenotypic variability that characterizes RyR1-related myopathies.

There is ample historical evidence for non-uniformity of function for RyR1 tetramers in studies of heterozygous MH and CCD mutant carriers [232–235]. Dr. Tom Nelson [232] detected 2 groups of SR Ca^{2+} release channels in skeletal muscle biopsies from 4 MH patients with unidentified mutations. Among 13 single channels recorded, 7 exhibited the functional activity of mutant channels, while 6 exhibited normal activity, corresponding to wt channels. Dr. Nirah Shomer [233] measured the function of isolated single channels from heterozygous R614C MH pig muscle, and classified them into 3 groups: wild-type homotetramer; mutant homotetramer; and mutant heterotetramer [234]. Dr. Gerhard Meissner [235] measured the single channel function of several transiently expressed CCD and MmD RyR1 mutants and confirmed that homotetrameric wild-type and mutant RyR1 channel functions were present, together with heterotetrameric channels of varying functional activity. For some RyR1 mutations, the presence of only one mutant subunit was sufficient to affect the properties of the tetramer, while for other mutations, more than one subunit was required [235]. In total, these studies show that functional variability is an intrinsic feature of the heterozygous expression of Ca^{2+} release channels in RyR1-related congenital myopathies and MH.

Studies of the lack of uniformity of Ca^{2+} release channel function in MH or CCD mutant RyRs have now been carried forward to the investigation of mouse models, in particular, the IT/+ model of CCD. The homozygous expression of the *Ryr1*^{I4895T} EC-uncoupled CCD mutation in a KI mouse line was neonatally lethal. However, the homozygous (IT/IT) mutant RyR1 subunits were expressed in normal amounts, assembled into tetramers and targeted correctly to the junctional SR, where they formed ordered arrays in precise juxtaposition with DHPR complexes [188]. IT/IT RyR1 was devoid of orthograde Ca^{2+} release channel activity, but retained the ability to support the retrograde entry of Ca^{2+} through its interaction with DHPR. Thus the loss of orthograde Ca^{2+} release function did not alter either the structural integrity of the RyR1 protein or the supramolecular organization of the CRU.

Heterozygous *Ryr1*$^{I4895T/+}$ (IT/+) mice survived the neonatal period. The earliest morphological abnormality seen in skeletal muscle from the IT/+ mouse line was a lack of uniformity of skeletal muscle contraction in very young mice (Fig. 2, upper panel) [204]. The mice then developed progressive core myopathy over the next 8 to18 months [204]. The phenotype was exhibited on a stable genetic background, thus minimizing genetic variability. Moreover, RT-PCR analyses revealed equimolar levels of expression of wt and mutant *Ryr1* transcripts, while western blotting experiments showed that total RyR1 protein content was unchanged by the mutation. In spite of these facts, there was variation in disease penetrance.

In a search for possible variant functions in heterozygous I4898T RyR1 channels [236], measurements of single channel activity revealed the co-existence of 3 functionally different groups of RyR1 tetramers: those with normal Ca^{2+} conductance (5 channels out of 20); those with a 12% decrease in Ca^{2+} conductance (6 out of 20); and those with no Ca^{2+} conductance (9 out of 20). The presence of three functional groups of RyR1 tetramers at a roughly equal ratio is inconsistent with the view that only 1/16 of the RyR1 molecules, containing only wt subunits, will be fully active; that 1/16 of the RyR1 molecules, containing only mutant subunits, will be inactive; and that 14/16, containing varying ratios of wt and mutant subunits, will have variable activities. It is, however, fully consistent with the assumption that the group with normal channel activity includes not only wt homotetramers, but also tetramers with 3 wt and 1 mutant subunits, for a cumulative fraction of 5/16; that the group of inactive channels includes not only mutant homotetramers, but also tetramers with 3 mutant and 1 wt subunits, for a cumulative fraction of 5/16; and that the group of channels with 12% decreased Ca^{2+} channel activity is formed by tetramers with 2 mutant and 2 wt subunits, for a cumulative fraction of 6/16. This deduced distribution of functional activity among various IT/+ tetramers is shown schematically in Fig. 4. For the specific case of I4898T/wt expression, a dark yellow "puff" of Ca^{2+} release illustrates 100% of full activity; a light yellow puff represents 88% of full activity and the lack of a puff indicates essentially 0 channel function for the different combinations of subunits resulting from heterozygous expression of this specific mutation.

These data support the view that there is functional heterogeneity among I4898T RyR1 tetramers. Indeed, the incorporation of IT mutant subunits into Ca^{2+} release channel tetramers results in a dominant-negative suppression of Ca^{2+} conductance that reduces the overall rate and magnitude of Ca^{2+} release, by 36% [236]. We propose a further layer of complexity: the random distribution of channels within each CRU harbors the potential to lead to both non-uniformity of Ca^{2+} release among CRUs and to non-uniformity of contraction among individual sarcomeres regulated by these individual CRUs. We have proposed that the resulting non-uniformity of sarcomeric contraction is the underlying cause of the variable disease phenotype in heterozygous IT/+ mice [204].

Our EM studies captured the ultrastructural consequences of the heterogeneity of contraction that exists within IT/+ myofibers [204]. The discordant contraction between adjacent myofibers is first seen as the disruption of sarcomeric register. Such discordance would create physical stresses within myofibers that would lead to tearing and shearing between myofibrils and to displacement of mitochondria and the sarcotubular and system that would, over time, cause physical and functional damage to Ca^{2+} regulatory, energetic and contractile systems As these processes progress, small foci of damage would coalesce and manifest as minicores, cores and rods that would compromise muscle function and force, further diminishing muscle strength. It is well established, in both human CCD patients and in IT/+ mice, that cores are not the primary cause of muscle weakness, since cores often form late in life, whereas muscle weakness usually manifests perinatally or during the first years of life [204–209]. However, cores may represent an aggravating factor and further compromise muscle strength in a progressive manner. Most importantly, the heterogeneity of Ca^{2+} release and contractile force would occur, regardless of whether the heterozygous RyR1 mutation acted by rendering the channel hypersensitive to stimuli (MH and some CCD mutations) or by interfering with Ca^{2+} conductance/gating/permeation (the majority of CCD mutations).

On the basis of these observations and deductions, we propose the unifying theory that the pathogenic mechanism leading to the formation of structural abnormalities and to clinical variability in MH, CCD and other RyR1-related muscle disorders is the differential release of Ca^{2+} from functionally disparate, heterozygous CRUs [204].

6. Conclusions

Research over the past 50 years on MH, CCD and CPVT has recorded steady progress in our understanding of their etiology and pathology. As one result, therapeutic agents such as dantrolene for MH and β-blockers for CPVT have been incorporated into medical practice. Through genetic testing, many individuals in MH and CPVT affected families can be identified and potentially fatal MH episodes or

arrhythmias can be anticipated and prevented. The generation of animal models for these diseases is well underway and analyses of these models will continue to provide insights into molecular processes leading to the various diseases. Such studies will also contribute to our understanding of the fundamental mechanisms of EC coupling and the principles of skeletal muscle contractility that function in health and disease. Ultimately, mouse model lines will offer the potential for testing therapeutic approaches that might help combat these disorders and diseases.

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