CALCIUM as a CELLULAR REGULATOR

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Pathology of Calcium-Transporting Membrane Systems

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Muscle contraction is regulated by the concentration of free Ca\(^{2+}\) in the sarcoplasm (Ebashz et al., 1969). In skeletal muscle, sarcoplasmic Ca\(^{2+}\) concentrations are controlled almost exclusively by the sarcoplasmic reticulum, so that skeletal muscle can be stimulated to contract repeatedly in the absence of extracellular Ca\(^{2+}\). Although the sarcoplasmic reticulum contains many proteins (Lytton and MacLennan, 1992), Ca\(^{2+}\) uptake in fast-twitch skeletal muscle is carried out by only one, the sarco- or endoplasmic reticulum Ca\(^{2+}\)-ATPase isoform, SERCA1, encoded by the \textit{ATP2A1} gene. Uptake of Ca\(^{2+}\) by slow-twitch skeletal muscle, cardiac muscle, smooth muscle, and nonmuscle tissues is through one or the other of two alternatively spliced SERCA2 isoforms encoded by the \textit{ATP2A2} gene. Ca\(^{2+}\) release from fast- and slow-twitch skeletal muscle is through the Ca\(^{2+}\)-release channel (sometimes referred to as the ryanodine receptor) isoform 1, encoded by the \textit{RYR1} gene, while Ca\(^{2+}\) release from cardiac muscle is through the ryanodine receptor isoform 2 (\textit{RYR2}). Defects in any of these genes would be expected to result in altered Ca\(^{2+}\) regulation and tissue-specific abnormalities. In the case of \textit{RYR1}, gene knockouts are lethal (Takeshima et al., 1994), reflecting its expression in more than one muscle type. It would be predicted that knockout of \textit{ATP2A2} would also be lethal, since it is expressed in many tissues. In this review chapter, we describe mutations in \textit{RYR1} that are not lethal, but which lead to malignant hyperthermia and central-core disease, and mutations in \textit{ATP2A1} that lead to Brody disease (BD).

Malignant Hyperthermia

Although malignant hyperthermia (MH) was first recorded in 1900 (Gibson et al., 1900), it was not until six decades later that Denborough et al. (1962) reported 10 cases in a single Australian family, demonstrating that MH is inherited as an autosomal dominant abnormality. By 1970, enough evidence had accumulated to assign the site of the primary defect to the skeletal muscle (Britt and Kalow, 1968; Berman et al., 1970). In the early 1970s, Kalow et al. (1970) showed that contracture of muscle fascicules from MH-susceptible (MHS) patients were more sensitive than normal to caffeine and Ellis et al. (1972) demonstrated similar high sensitivity of MH muscle to halothane. These abnormal responses provided a diagnostic test for MH — the caffeine–halothane contracture test (CHCT) — that is still widely used. In 1966, it was discovered that pigs afflicted with porcine stress syndrome (PSS) also developed MH reactions that were virtually identical to human MH reactions, thus providing an animal model for human MH (Hall et al., 1966). In 1975, dantrolene was demonstrated to be an antidote for MH reactions, permitting full recovery in many cases (Harrison, 1975). In recent years, genetic analysis has shown that a large fraction of human MH cases, but not all, are associated with dominant mutations in the \textit{RYR1} gene that encodes the Ca\(^{2+}\)-release channel of skeletal muscle sarcoplasmic reticulum (the ryanodine receptor) (MacLennan and Phillips, 1992). Current research is aimed at the identification of all
genes and all mutations that cause human MH. Excellent comprehensive reviews of clinical aspects of MH and its physiological, biochemical, and genetic basis have been published elsewhere (Britt, 1991; Mickelson and Louis, 1996).

Human Malignant Hyperthermia

Human MH is a genetic abnormality, inherited as an autosomal dominant mutation (Denborough et al., 1962). Britt and Kalow (1970) estimated the incidence to be about 1 case in 15,000 anesthetics in children and about 1 case in 50,000 to 1 in 100,000 anesthetics in adults. Ørding (1983) found the incidence of MH episodes to be about 1 case in 65,000 anesthetics in Denmark. These reported incidences are probably marked underestimates of the true incidence of MH susceptibility because of the incomplete penetrance of the gene and the difficulty in defining mild reactions. Many MH-susceptible patients are never identified because they are never anesthetized or are not anesthetized enough times to develop an MH reaction. An MH-like episode may occur in individuals who have inherited muscle diseases with deleterious phenotypes, such as central-core disease (CCD, Denborough et al., 1973), King-Denborough syndrome (King and Denborough, 1973), Duchenne muscular dystrophy (Brownell et al., 1983), myotonia fluctuans (Vita et al., 1995), and possibly other myopathies (Brownell, 1988; Heiman-Patterson et al., 1988). It is, therefore, important to differentiate true MH from those muscle diseases that give rise to MH-like reactions (Iaizzo and Lehmann-Horn, 1995).

Individuals with MH susceptibility may respond to the administration of potent inhalational anesthetics and depolarizing skeletal muscle relaxants with a rising end tidal carbon dioxide, skeletal muscle rigidity, tachycardia, unstable and rising blood pressure, hyperventilation, cyanosis, a falling arterial oxygen tension, an increasing arterial carbon dioxide tension, lactic acidosis, and, eventually, fever (Britt, 1991). Muscle cell damage brings about electrolyte imbalance, with early elevation of serum K+ and Ca2+ and a later elevation of muscle enzymes, such as creatine kinase, and muscle proteins, such as myoglobin in the serum and urine. If therapy is not initiated immediately, the patient may die within minutes from ventricular fibrillation, within hours from pulmonary edema or coagulopathy, or within days from postanoxic neurological damage and cerebral edema or obstructive renal failure, resulting from the release of muscle proteins into the circulation (Britt, 1991). During convalescence, muscle soreness and muscle edema may develop: In some instances, the reactions induced in susceptible individuals may be much milder (Britt, 1991), being characterized by masseter muscle rigidity alone (Kostko et al., 1992), by fever alone, or by reactions of intermediate severity.

Malignant hyperthermia crises are rare below the age of 3 years and the incidence of crises declines progressively above the age of 30 years. Males are more affected, both in terms of incidence of reactions and of positive biopsies (Britt, 1991), possibly because of their greater musculature or because of hormonal differences. Many individuals who have had MH reactions have had multiple previous uneventful general anesthetics, often of long duration and with known triggering agents. The reason for the failure of a crisis to be precipitated on exposure to potent inhalational anesthetics and/or to succinylcholine is not clear, but it may be that an additional environmental trigger, such as strenuous exercise or extreme emotional stress and agitation (both of which might affect circulating hormonal levels), a major muscle injury, drugs, or a viral or bacterial infection associated with fever, is needed to initiate a reaction.

Anesthesiologists identify many patients at risk through case histories, which include information on their kinship to individuals who have had an MH reaction and/or a combined history of a persistently elevated creatine kinase with chronic and inacapacitating muscle pain and cramps. For patients known or suspected to have malignant hyperthermia, anesthetic routines are changed to any desired combination of nontriggering anesthetics, such as barbiturates, tranquilizers, narcotics, propofol, ketamine, nitrous oxide, and local anesthetics (Britt, 1991; McKenzie et al., 1992).

Under present-day standard anesthetic practice, heart rate, blood pressure, body temperature, end tidal carbon dioxide production, and arterial oxygen saturation are monitored during the course of anesthesia (Britt, 1991). An increase in several of these factors may lead to the clinical diagnosis of MH (Larach et al., 1994). When this occurs, the administration of the MH-triggering anesthetic is stopped immediately, the gas machine is changed and the patient is hyperventilated with 100% oxygen, and the antidote — dantrolene — is administered until muscles relax, fever, heart rate and respiratory rate decline significantly toward normal, and blood gases normalize. Sodium bicarbonate is also given to correct metabolic acidosis, insulin is given if serum potassium and blood glucose are elevated, and furosemide and mannitol are given to prevent the onset of acute renal failure or muscle and brain edema. These practices have lowered the death rate from MH episodes from over 80% to less than 7% in recent years, but neurological, muscle, and kidney damage still contribute to the morbidity that results from MH reactions (Britt, 1991).
Diagnostic Tests for Malignant Hyperthermia

In humans, MH susceptibility is seldom associated with ill health. Accordingly, the most important goals of MH research are to provide therapy during an acute MH episode and to prevent MH episodes through identification of MHS individuals in advance of anesthesia.

The caffeine–halothane contracture test (CHCT) and the in vitro contracture test (IVCT) were developed on the premise that the muscle from MHS individuals might be abnormally sensitive to agents that induce contractures. Therefore, MHS muscle fascicles might contract in the presence of lower amounts of either caffeine or halothane, or, conversely, have larger contractures in the presence of these agents than the muscle from normal individuals. The North American test protocol (Larach, 1989) and the European test protocol (European MH Group, 1984) have been standardized on the basis of this premise. In both protocols, a positive response to both caffeine and halothane results in the diagnosis of MHS. A positive response to either caffeine or halothane, but not both, is considered to be MHS in the North American protocol, but as MH-equivalent (MHE) in the European test. The MHE patients are treated as MHS patients.

The CHCT is a useful clinical test (Larach, 1993), distinguishing those at risk for MH from unaffected patients. As a clinical test, the CHCT ensures that appropriate anesthetics are administered to those patients who are MHS, while those diagnosed as normal can be treated with routine anesthetics. Since failure to detect MH susceptibility can result in a serious or fatal outcome, sensitivity (the ability of the test to detect MH when it is present) approaching 100% is more important for a clinical diagnosis than specificity (the ability of the test to exclude false positive results in normal patients). Strict, objective criteria have been developed to define when an MH reaction has almost certainly occurred (D6), when it has very likely occurred (D5), when it is unlikely to have occurred (D2), or likely not to have occurred: (D1) (Larach et al., 1994). The clear definition of an acute MH reaction has permitted case findings for determination of the sensitivity of the CHCT and IVCT. Testing of control patients has allowed the determination of specificity. The North American CHCT currently achieves 97% sensitivity and 78% specificity (95% CI, 70–85%) based on 32 MH patients with a D6 MH reaction and 120 controls (95% CI, 84–100%) (Larach et al., 1992a, 1992b; Allen et al., 1998). The European IVCT offers 99% sensitivity (95% CI, 84–100%) based on investigation of 17 patients who survived a D6 MH reaction and 28 controls and 93.6% specificity (Ording et al., 1997). Combined results from the European MH group suggest that the IVCT has 100% sensitivity and 87% specificity (95% CI, 82–92%) on the basis of studies of 95 patients with a D6 reaction and 200 controls.

It is most likely that limitations in sensitivity and specificity of the CHCT arise because the outcome of the test depends on the interplay among a very large number of biochemical reactions within muscle cells. In a multifactorial system, the potentially deleterious effect of an abnormal Ca2+-release channel, the most common cause of MH, may be compensated for in some individuals by overactivity of systems such as the sarcoplasmic reticulum Ca2+ pump, the plasma membrane Ca2+ pump, the Na+/Ca2+ exchangers, or the Ca2+-uptake system of the mitochondria, all of which might act to bring about Ca2+ homeostasis before contracture is triggered. These factors might be altered further by the fitness of the muscle analyzed.

Porcine Malignant Hyperthermia

Individuals among herds of lean, heavily muscled swine are susceptible to fatal crises characterized by muscle rigidity, mottling of the skin, and tachycardia (O'Brien, 1987). This syndrome is referred to as the porcine stress syndrome (PSS). Crises are brought on by physical and emotional stresses, including overheating, exercise, mating, transportation to market, and fear. When given halothane and succinylcholine, or halothane alone, susceptible pigs frequently develop MH reactions similar to those suffered by human MHS patients (Hall et al., 1966).

Stress-induced deaths in swine occur predominantly with homozygous MH animals and rarely in heterozygotes. In a study of the response of homozygotes and heterozygotes to a 3-minute halothane challenge, none of 197 heterozygotes responded, while 168 of 179 homozygotes responded to the halothane challenge (Otsu et al., 1991). In an early study (Britt et al., 1978), continued halothane administration was found to bring about increased heart rate, cyanosis, rigidity, and fever in both MH homozygotes and heterozygotes. The response was graded from normal through MH heterozygotes to MH homozygotes. In a more recent study (Fletcher et al., 1993), however, heterozygotes did not respond to continued halothane administration, even in the presence of succinylcholine, raising the possibility that the genetic background on which the MH gene is expressed may affect the MH response. The MH heterozygotes gain about 5% more lean meat than normal animals (Simpson and Webb, 1989; O'Brien et al., 1994), they have better feed conversion and fat is redistributed from muscle to backfat.
PATHOLOGY OF CALCIUM-TRANSPORTING MEMBRANE SYSTEMS

The Physiological Basis for Malignant Hyperthermia

Examination of the early and late events in the development of a halothane-induced MH reaction in swine have proved very informative (Berman et al., 1970). Studies of blood chemistry revealed that lactic acidosis, presumably originating in glycogenolysis and glycolysis, and the release of K\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\) from muscle occurs within seconds after halothane administration. Observed rises of serum K\(^+\) to as high as 7–8 mM have been sufficient to cause cardiac arrest in some cases. A rise in body temperature usually occurs only after 15 or 20 minutes, and perhaps even substantially longer. On the basis of measurements of oxygen uptake, the major source of heat production has been shown to be anaerobic, arising from the breakdown of creatine phosphate and ATP (Berman et al., 1970). The neutralization of bicarbonate in the blood by lactic acid and the direct production of carbon dioxide by the tetracarboxylic acid cycle produces large amounts of CO\(_2\) in the blood and exhaled air. The release of Ca\(^{2+}\) into the blood from the muscle was the first indication of a disturbance in Ca\(^{2+}\) homeostasis in muscle was the primary defect in MH.

Studies of the mechanisms controlling changes in muscle tension have revealed that the interaction of actin and myosin is regulated by Ca\(^{2+}\) and that Ca\(^{2+}\) regulation is mediated through the Ca\(^{2+}\)-binding protein, troponin (Zot and Potter, 1987). Muscle Ca\(^{2+}\) concentrations are regulated by the activities of Ca\(^{2+}\) pumps and channels located in the sarcoplasmic reticulum and transverse tubular systems. The Ca\(^{2+}\) also plays a role in the control of glycolysis in muscle through its activation of phosphorylase kinase (Brostrom et al., 1971). A defect in Ca\(^{2+}\) regulation, leading to the chronic elevation of Ca\(^{2+}\) within the sarcoplasm, could induce muscle contracture, extensive glycolysis, and enhanced mitochondrial oxidation of glycolytic end products (Fig. 1). These various reactions, leading to high turnover of ATP, could be responsible for the elevated temperatures associated with MH episodes (Britt, 1991; MacLennan and Phillips, 1992).

Abnormalities in the Ca\(^{2+}\) pump were ruled out in biochemical studies of MH swine (Nelson, 1988). It is of interest, however, that muscle from Brody patients with Ca\(^{2+}\)-pump deficiency gave a positive in vitro CHCT result (Karpatic et al., 1986), even though a number of other abnormalities in MH reactions have not been reported in Brody disease. On the other hand, higher rates of Ca\(^{2+}\)-induced Ca\(^{2+}\) release, particularly at low levels of inducing Ca\(^{2+}\), were observed in membrane vesicle preparations from both human (Endo et al., 1983) and porcine (Ohnishi et al., 1983) muscle, and closure of single porcine MH channels at high Ca\(^{2+}\) concentrations was shown to be inhibited (Fill et al., 1990; Shomer et al., 1993). In comparable studies of human muscle, Ca\(^{2+}\)-release channels with abnormally high caffeine sensitivity were detected in MH individuals (Fill et al., 1991). In sarcoplasmic reticulum from MHS swine, ryanodine binding, which is dependent on the open state of the Ca\(^{2+}\)-release channel, was found to be enhanced (Mickelson et al., 1988). Digestion with trypsin revealed an alteration in the amino acid sequence of the Ca\(^{2+}\)-release channel in MHS animals (Kudson et al., 1990). Thus, comparative biochemical and physiological studies implicated the Ca\(^{2+}\)-release channel as a potential causal factor for MH.

The Ca\(^{2+}\)-release channels have been cloned from skeletal (RYR1) (Takeshima et al., 1989; Zorzato et al., 1990; Fujii et al., 1991), cardiac (RYR2) (Nakai et al., 1990; Otsu et al., 1990), and nonmuscle sources (RYR3) (Giammini et al., 1992; Hakamata et al., 1992). These proteins contain from 4872 to 5037 amino acids with masses between 550,000 and 564,000 Da. The Ca\(^{2+}\)-release channel has been isolated from skeletal muscle and shown to form a tetrameric complex (Inui et al., 1987). Transmembrane sequences are located at the COOH-terminal end of the molecule (Takeshima et al., 1989; Zorzato et al., 1990). Predicted ATP- and Ca\(^{2+}\)-dependent calmodulin-binding sites are clustered in two sites in the molecule. One lies in the proposed transmembrane sequences in the COOH-terminal end of the protein, encompassing the region between amino acid residues 4255 and 4499 (Takeshima et al., 1989). A second regulatory region has been proposed to lie between amino acid residues 2600 and 3000 (Otsu et al., 1990). This sequence would contain residues 2809 and 2843 in cardiac and skeletal isoforms, respectively, the major phosphorylation sites in these proteins (Witche et al., 1991; Suko et al., 1993). A predicted ATP-binding sequence begins at residue 2652 and the region between residues 2800 and 3050 contains predicted calmodulin-binding sites (Otsu et al., 1990).

A defect in the Ca\(^{2+}\)-release channel, giving rise to abnormal Ca\(^{2+}\) regulation within skeletal muscle, could account for all of the signs of MH (MacLennan and Phillips, 1992). If the Ca\(^{2+}\)-release channels had longer open times in the presence of anesthetic agents, intracellular Ca\(^{2+}\) might be chronically elevated, resulting in muscle contracture and activation of the first steps in glycogenolysis through activation of phosphorylase kinase. Muscle contracture and the pumping of excessive amounts of cytoplasmic Ca\(^{2+}\) back to the lumen of the sarcoplasmic reticulum would consume large amounts of ATP, thus generating large quantities of heat. The ADP formed would stimulate glycolysis and the mitochondrial oxidation of pyruvate derived from glucose. These hypermetabolic responses would lead to deple-
Figure 27.1 A proposed mechanism for the induction of malignant hyperthermia caused by abnormalities in the Ca\(^{2+}\)-release channel of skeletal muscle sarcoplasmic reticulum. In a normal relaxation/contraction cycle (left), Ca\(^{2+}\) is pumped into the sarcoplasmic reticulum by a Ca\(^{2+}\) pump to initiate relaxation, stored within the lumen in association with calsequestrin, and released through a Ca\(^{2+}\)-release channel to initiate contraction. Glycolytic and aerobic metabolism proceed only rapidly enough to maintain the energy balance in the cell. The Ca\(^{2+}\) release is highly regulated and, even when stimulated, has a relatively short open time. Abnormal MH or CCD Ca\(^{2+}\)-release channels (right) are sensitive to lower concentrations of activators of opening, releasing Ca\(^{2+}\) at enhanced rates, and not closing readily. The activity of the mutated channel floods the cell with Ca\(^{2+}\) and overpowers the Ca\(^{2+}\) pump that would normally lower cytoplasmic Ca\(^{2+}\). Sustained muscle contraction (contracture) accounts for rigidity, while sustained glycolytic and aerobic metabolism account for the generation of excess lactic acid, CO\(_2\) and heat, and for enhanced oxygen uptake. Damage to cell membranes and imbalances of ionic concentrations can account for the life-threatening systemic problems that arise during a malignant hyperthermia episode. (Adapted from MacLennan, D. H. and Phillips, M. S. (1992) Science 256: 789, with permission, © AAAS.)

The production of ATP, glycogen, and oxygen; to the production of excess lactic acid, carbon dioxide, and heat; and, ultimately, to the disruption of intracellular and extracellular ion balances, with consequent muscle cell damage.

Linkage of the RYR1 Gene to Malignant Hyperthermia

In early studies of porcine MH, Andersen and Jensen (1977) demonstrated linkage between inheritance of porcine MH and polymorphisms in the gene that encodes glucose phosphate isomerase (GPI). Later studies established a linkage group for the porcine HAL gene (the designation of the MH gene that gives rise to halothane sensitivity), GPI, and the gene for 6-phosphogluconate dehydrogenase (PGD) (Archibald and Imlah, 1985; Ganhe and Juneja, 1985), located near the centromere of pig chromosome 6 (Davies et al., 1988; Chowdhary et al., 1989; Harbitz et al., 1990). The homologous region around the human GPI locus was found to be on the long arm of chromosome 19 (Lusis et al., 1986), making this a candidate region for human MH. Cloning
of the human skeletal muscle ryanodine receptor (RYR1) cDNA (Zorzato et al., 1990) led to the localization of RYR1 to human chromosome 19q13.1 (MacKenzie et al., 1990) and permitted the identification of several restriction fragment length polymorphisms (RFLPs) in the human RYR1 gene (MacLennan et al., 1990). In a study of linkage between inheritance of MH and these RYR1 polymorphisms and flanking markers, cosegregation with RYR1 markers was found in 23 meioses in nine families, leading to a lod score (the logarithm of the odds favoring linkage vs. nonlinkage) of 4.2 for a recombinant fraction of 0.0. The probability of linkage of more than 10,000:1 identified RYR1 as a candidate gene for MH in humans (MacLennan et al., 1990). In an independent investigation of linkage of human MH to a series of chromosome 19q markers, the MH locus was also assigned to the region of human chromosome 19 where RYR1 was localized (McCarthy et al., 1990).

Malignant Hyperthermia Mutation in Swine

The demonstration of linkage of RYR1 to MH led to parallel searches in both swine and humans for sequence differences in the RYR1 gene between MH and normal individuals (Fuji et al., 1991; Otsu et al., 1991; Gillard et al., 1992a, 1992b). In a comparison of the cDNA sequences of MH (Pietrain) and normal (Yorkshire) pigs, the substitution of T for C at nucleotide 1843, leading to the substitution of Cys for Arg at amino acid residue 615, was the only amino acid sequence alteration found (Fuji et al., 1991). Initial studies showed an association between inheritance of the mutation and inheritance of MH in some 80 animals from five different breeds. Tight linkage was established in a study of backcrosses between British Landrace heterozygous animals of the N/j genotype and homozygous MH animals of the n/n genotype (Otsu et al., 1991) in which 376 animals were tested, including 338 that represented informative meioses. Cosegregation of the phenotype with the Cys for Arg substitution was observed, leading to a lod score favoring linkage of 102 for a recombinant fraction of 0.0.

The porcine MH mutation seems to have been selected because it contributes to dressed carcass weight, to leanness and heavy muscling, and to the redistribution of fat from muscle to backfat (Simpson and Webb, 1989; O'Brien et al., 1994). A possible explanation for these phenomena is that a leaky or hypersensitive Ca\(^{2+}\)-release channel could give rise to spontaneous muscle contractions and that continual toning of the muscle might lead to muscle hypertrophy (MacLennan and Phillips, 1992). The utilization of ATP for spontaneous contractions would limit the deposition of fat.

Malignant Hyperthermia Mutations in Humans

The demonstration of linkage between MH and a substitution of Cys for Arg in the RYR1 gene in swine led to a search for the corresponding mutation in human MH families. The equivalent human mutation, Cys for Arg, was first found in a single family of five members in which the mutation segregated with MH (Gillard et al., 1992a). This mutation has been found in about 4% of MH families worldwide. In most families where it has been found, it segregates with individuals who have been diagnosed by the CHCT as being MHS (Gillard et al., 1992a; Hogan et al., 1992), but in a few families where it is found, linkage was not observed (Deufel et al., 1995; Serfas et al., 1996). The questions that arise, therefore, are whether the Arg to Cys mutation is really causal of MH, whether the phenotypic diagnosis based on CHCT is incorrect, or whether other MH-causing mutations are also cosegregating in these families (MacLennan, 1995).

The assessment of the Arg to Cys mutation as causative of human MH has a strong genetic and biochemical basis. The corresponding mutation is linked to porcine MH with a lod score of 102 favoring linkage for a recombinant fraction of 0.0 (Otsu et al., 1991). The mutation has been found across a species barrier between swine and humans, where it segregates with MH in all but a few individuals analyzed (Gillard et al., 1992a). In biochemical studies of the porcine channel, a measurable defect has been observed in the closing of the Ca\(^{2+}\)-release channel (Fill et al., 1990; Shomer et al., 1993). When expressed in heterologous cell culture, the mutant form of the ryanodine receptor has been shown to release Ca\(^{2+}\) in response to lower levels of added caffeine or halothane than the normal channel (Otsu et al., 1994; Treves et al., 1994). All of these test results support the view that the Arg to Cys mutation is causal of MH.

The phenotypic assay (CHCT) has an inherent error, defined by its imperfect sensitivity and specificity. The limited specificity can probably be attributed to the multifactorial nature of the contracture response. Accordingly, the occasional discordance between the CHCT- and DNA-based diagnoses, at least for the Arg to Cys mutation, is most likely to arise from the CHCT test. Another possibility is that discordant results arise from the segregation of a second MH mutation in the family under study. These problems are complicating factors in proving the causal nature of other MH mutations through linkage of inheritance of MH and mutant RYR1 alleles (Phillips et al., 1994; MacLennan, 1995; Serfas et al., 1996).
Table 27.1 Mutations Associated with MH, CCD, or BD

<table>
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<th>Mutation</th>
<th>Region</th>
<th>Association</th>
<th>Reference(s)</th>
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<tr>
<td>RYR1 Mutations Associated with MH and CCD</td>
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<tr>
<td>Cys35Arg</td>
<td>Exon 2</td>
<td>MH</td>
<td>Lynch et al. (1997)</td>
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<tr>
<td>Arg163Cys</td>
<td>Exon 6</td>
<td>MH, CCD</td>
<td>Quane et al. (1993)</td>
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<tr>
<td>Gly248Arg</td>
<td>Exon 9</td>
<td>MH</td>
<td>Gillard et al. (1992b)</td>
</tr>
<tr>
<td>Gly341Arg</td>
<td>Exon 11</td>
<td>MH</td>
<td>Quane et al. (1994b)</td>
</tr>
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<td>Ile403Met</td>
<td>Exon 12</td>
<td>MH, CCD</td>
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<tr>
<td>Tyr522Ser</td>
<td>Exon 14</td>
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<td>Arg614Cys</td>
<td>Exon 17</td>
<td>MH</td>
<td>Gillard et al. (1992a)</td>
</tr>
<tr>
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<td>Exon 39</td>
<td>MH</td>
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<td>Phillips et al. (1994)</td>
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<td>Arg2438Cys</td>
<td>Exon 46</td>
<td>MH</td>
<td>Manning et al. (1998a)</td>
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<td>Exon 46</td>
<td>MH</td>
<td>Manning et al. (1998a)</td>
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ATP2A1 Mutations Associated with BD

<table>
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<th>Mutation</th>
<th>Region</th>
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<th>Reference(s)</th>
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<td>Cys67stop</td>
<td>Exon 15</td>
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<td>Defective splice donor</td>
<td>Intron 3</td>
<td>BD</td>
<td>Odermatt et al. (1996)</td>
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<tr>
<td>Frameshift at Pro${}^{47}$</td>
<td>Exon 5</td>
<td>BD</td>
<td>Odermatt et al. (1997)</td>
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Continued analysis of the DNA sequence of ryanodine receptors from probands from MH families has now associated MH with at least 15 RYR1 mutations, accounting for the abnormality in about 25% of MH families (Table 27.1). Thus, the causal MH mutations in about 75% of MH families have yet to be found.

The search for additional MH mutations in RYR1 is hampered by the large size of the gene. The cDNA is over 15,000 nucleotide base pairs (bp) long and the gene from which it is derived is 159,000 bp long (Phillips et al., 1996). The gene contains 106 exons, of which two are alternatively spliced into mRNA to create different forms of the protein. The MH mutations found to date have clustered in exons 2–18 and 39–46 (Table 27.1). If additional mutations continue to cluster in a few exons, mutant searches will be much less onerous than might be anticipated, considering the length of the coding sequences in the gene. Searching for Additional MHS Loci

The RYR1 gene is a very strong candidate gene for MH, but it is not the only one. There are cases where diagnosis appears to be accurate and where no linkage between RYR1 and MH can be defined (Ball and Johnson, 1993; MacLennan, 1995). In at least some of these cases, both false positive and false negative diagnoses would have to be invoked to prove linkage. Linkage of MH to chromosome 17q in several families has been reported (Levitt et al., 1992), making candidates of the sodium channel α-subunit gene (SCN4A) and two subunits of the dihydropyridine receptor, CACNL1B1 and CACNL1D1, located on chromosome 17q11.2–q24 (Olckers et al., 1992). In subsequent studies, however, linkage of MH to chromosome 17q and to the candidate genes was ruled out in other non-chromosome-19-linked European families (Sudbrak et al., 1993). It is possible that myotonia fluctuans and other demonstrated
defects of the sodium channel α-subunit protein might have been associated with abnormal responses to succinylcholine, including muscle rigidity (Iaizzo and Lehmann-Horn, 1995; Vita et al., 1995).

Malignant hyperthermia has also been linked to chromosome 7q, with a lod score less than 3 in a single family (Iles et al., 1994). The presence of the gene that encodes the α2δ subunit of the dihydropyridine receptor (CACNL1A3) on chromosome 7q21-q22 (Powers et al., 1994) establishes it as a candidate gene for MH. Sequencing of this candidate has not revealed a causal mutation.

Several large, nonchromosome-19-linked European MH families have been included in a systematic linkage study using a set of polymorphic microsatellite markers that cover the entire human genome (Sudbrak et al., 1995). A single family was linked to chromosome 3q13.1 with a lod score of 3.22. The high lod score found in this linkage analysis suggests that true heterogeneity exists for MH. Thus, this chromosome-3-linked family offers an excellent opportunity for identifying an additional causal gene in MH.

In continued analysis, two more candidate loci were identified (Robinson et al., 1997). The first is on human chromosome 1q, the site of a candidate gene, CACNL1A3, that encodes the α2δ-subunit of the dihydropyridine receptor. The second is on human chromosome 5p, where no candidate gene has been mapped. A third family provides evidence for at least one other unspecified locus.

Analysis of the chromosome-1q-linked family led to the discovery of the mutation of G3333 to A in the CACNL1A3 gene, leading to the mutation of Arg^{1086} to His in the α2δ-subunit of the L-type voltage-dependent Ca^{2+} channel (Monnier et al., 1997). This study clearly demonstrates the involvement of the dihydropyridine receptor in the regulation of the ryanodine receptor and illustrates how defects in dihydropyridine receptor function can manifest as defects in Ca^{2+} regulation, leading to susceptibility to malignant hyperthermia.

Central-Core Disease

Central-core disease (CCD) is a rare, nonprogressive myopathy, presenting in infancy, which is characterized by hypotonia and proximal muscle weakness (Shy and Magee, 1956). Additional variable clinical features include pes cavus, kyphoscoliosis, foot deformities, congenital hip dislocation, and joint contractures (Shuaib et al., 1987). Although signs may be severe, up to 40% of patients who demonstrate central cores may be clinically normal (Shuaib et al., 1987). Diagnosis is made on the basis of the lack of oxidative enzyme activity in central regions of skeletal muscle cells (Dubowitz and Pearse, 1960), observed upon histological examination of muscle biopsies. Electron microscopic analysis shows dismantling of the contractile apparatus, ranging from blurring and streaming of the Z-lines to total loss of myofibrillar structure (Dubowitz and Roy, 1970; Issacs et al., 1975; Hayashi et al., 1989). The sarcolemmal reticulum and transverse tubular systems are greatly increased in content and are, in general, less well structured. The NADH-tetrazolium reductase reactions reveal pale circular areas referred to as central cores. Mitochondria are depleted in the cores, but may be enriched around the surfaces of the cores. The inheritance of CCD has been linked closely to RYRI (Kausch et al., 1991; Mulley et al., 1993) and to four RYRI mutations in several CCD families (Quane et al., 1993; Zhang et al., 1993). The physiological changes in muscle cells that give rise to the presence of central cores is of current research interest.

The Genetic Basis of Central-Core Disease

Genetic analysis indicates that the disorder is inherited as an autosomal dominant trait with variable penetrance (Issacs et al., 1975; Eng et al., 1978; Byrne et al., 1982; Hayashi et al., 1989). An important feature of CCD is its close association with susceptibility to malignant hyperthermia (Denborough et al., 1973; Eng et al., 1978; Frank et al., 1980; Brownell, 1988). This association led investigators to establish a linkage between CCD and markers in chromosome 19q in large Australian (Haan et al., 1990; Mulley et al., 1993) and European (Kausch et al., 1991) CCD pedigrees. Zhang et al. (1993) linked the substitution of Arg^{264} with His to CCD in a Canadian family, obtaining a lod score of 4.8 favoring linkage with a recombinant fraction of 0.0, while Quane et al. (1993) associated CCD with three other RYRI mutations (Table 27.1).

Mutations corresponding to fifteen human MH and CCD mutations were made in a full-length rabbit RYRI cDNA, and wild type and mutant cDNAs were transfected into HEK-293 cells (Tong et al., 1997). After about 48 hours, intact cells were loaded with the fluorescent Ca^{2+} indicator, fura-2, and intracellular Ca^{2+} release, induced by caffeine or halothane, was measured by photometry. Ca^{2+} release in cells expressing MH or CCD mutant ryanodine receptors was invariably significantly more sensitive to low concentrations of caffeine and halothane than Ca^{2+} release in cells expressing wild-type receptors or receptors mutated in other regions of the molecule. Abnormal sensitivity in the Ca^{2+} photometry assay provides supporting evidence for a causal role in MH and CCD for each
of fifteen single amino acids mutations in the ryanodine receptor.

It is of interest that 8 of the 12 codons that have been shown to give rise to 15 MH or CCD mutations (Table 27.1) encode amino acids lying between Cys35 and Arg614, while the other four codons encode amino acids lying between Arg2162 and Arg2458. Thus, there are two clusters of MH mutations in the 5000-amino acid protein that may constitute the MH regulatory domains. In each of these two regions, MH and CCD mutations are interspersed, demonstrating that there is no preferential site in the ryanodine receptor where mutation might give rise to the formation of central cores (Table 27.1). An interesting feature of the known MH and CCD mutations is that 13 of the 15 involve either loss or gain of an Arg residue. This suggests that positive charges within the two proposed regulatory domains are critical to regulatory function.

Arginine-614, which gives rise to two MH mutants (Table 27.1), lies in a region of the ryanodine receptor that is homologous to the inositol triphosphate (IP3)-binding region in the IP3 receptor (Mignery and Stühlf, 1990; Miyawaki et al., 1991). This may indicate that the cluster of amino acids between residues 35 and 614 forms a regulatory domain in the Ca2+-release channel that is concerned with ligand activation of the channel. The MH and CCD mutations between Arg2162 and Arg2458 may form a second MH regulatory domain. The boundaries of these two proposed regulatory domains might be deduced from analysis of the alignments of the amino acid sequences of the ryanodine and IP3 receptors. They are contiguous between amino acids 1 and 668 (of the ryanodine receptor), but the next 967 amino acids in the ryanodine receptor are deleted from the IP3 receptor. The amino acid sequences are also contiguous between amino acids 1637 and 2650 of the ryanodine receptor, but, at this point, another 1045 amino acids are deleted from the IP3 receptor, so that contiguity is re-established only after amino acid 3695 of the ryanodine receptor. The MH and CCD mutations lying between Cys35 and Arg614 fit nicely into the first conserved regulatory domain (amino acids 1–668) and mutations lying between Arg2162 and Arg2458 fit nicely into the second conserved domain (amino acids 1637–2650). Thus, the boundaries of these two conserved domains may prove to be the boundaries within which MH and CCD mutations, which affect regulation of the Ca2+-release channel, will be found. The sequence that encompasses amino acid residues 2600–3000, previously proposed to include ligand-binding sites (Otsu et al., 1990), may represent a third regulatory domain that is unique to ryanodine receptors.

The Physiological Basis for Central-Core Disease

The Arg615 to Cys mutation is associated with muscle hypertrophy in swine, while the Arg234 to His mutation, for example, is associated with variable degrees of muscle atrophy, metabolically inert cores, proximal muscle weakness, and MH (Table 27.1). If both of these mutations were to lead to poorly regulated Ca2+ release into the muscle cell, they could trigger spontaneous muscle contractions. Such spontaneous contractions could lead to the muscle hypertrophy observed in swine. In this case, the system of pumps and exchangers in the plasma membrane and organellar systems of mitochondria and sarcoplasmic reticulum within the cell could remove excess Ca2+ from the sarcoplasm without deleterious effects on the muscle cell. The CCD mutations might be more severe, leading to disorganization of the contractile proteins in the central core, a proliferation of sarcoplasmic reticulum and transverse tubules, and a loss of functional mitochondria, leading to damage to the interior of the cell and to loss of mitochondrial function and structural abnormalities in the central core (Fig. 2). These losses could, in turn, lead to muscle weakness and atrophy.

The physical alterations in the central core may be the result of physiological adaptation to functional alterations in the Ca2+-release channel that lead to elevated Ca2+ levels within the muscle cell. Muscle cells regulate Ca2+ through at least four systems: plasma membrane Ca2+ pumps (PMCA), sarco(endo)plasmic reticulum Ca2+ pumps (SERCA), Na+/Ca2+ exchangers, and mitochondria. The Ca2+ pumps and Na+/Ca2+ exchangers in the plasma membrane can remove Ca2+ from the muscle cell, depositing it in extracellular spaces (Carafoli, 1987). Of these two systems, the Ca2+ pump has the higher affinity for Ca2+, while the Na+/Ca2+ exchanger is more active with elevated levels of intracellular Ca2+. The sarcolemmal reticulum is the major regulator of Ca2+ within the muscle cell, removing it from the cytoplasm, storing it, and releasing it again to initiate muscle contraction. If Ca2+ concentrations are elevated, the mitochondria can transport Ca2+ to matrix spaces, thereby protecting the cell from Ca2+-induced damage (Wrogemann and Pena, 1976). If the Ca2+-release channel were to release excessive amounts of Ca2+ within the muscle cell, then the Na+/Ca2+ exchanger and mitochondria might play a more important role in Ca2+ regulation in a CCD cell than in a normal cell. Extrusion of excess Ca2+ from the cell might, itself, have deleterious effects on the skeletal muscle cell, which is believed to carry out intracellular cycling of a constant level of Ca2+ without uptake and expulsion of external Ca2+. 
Figure 27.2 A proposed mechanism for the differential phenotypic effects of different MH and CCD mutations. (A) The MH mutations in the ryanodine receptor lead to the common phenotype of sensitivity to anesthetics. (B) Some mutations may also lead to spontaneous Ca\(^{2+}\) release sufficient to trigger spontaneous contractions. If this trigger Ca\(^{2+}\) were readily regulated, the major phenotypic effect would be spontaneous exercise-induced muscle hypertrophy. The Arg\(^{615}\) to Cys mutation was selected in swine because it leads to increased lean muscle mass. (C) Mutations leading to excessive spontaneous Ca\(^{2+}\) release may have no phenotypic effect on the periphery of the cell, but be deleterious to the central core. The Ca\(^{2+}\) released from the sarcoplasmic reticulum can be regulated by four systems, including the organellar sarcoplasmic reticulum and mitochondria and the plasma membrane Ca\(^{2+}\) pumps and Na\(^{+}\)/Ca\(^{2+}\) exchangers. Under normal circumstances, the bulk of the Ca\(^{2+}\) is cycled only through the sarcoplasmic reticulum. If enhanced Ca\(^{2+}\) release occurred spontaneously, in CCD muscle, the additional Ca\(^{2+}\) regulatory systems might be co-opted to regulate Ca\(^{2+}\). The plasma membrane exchangers and pumps would be effective in regulating Ca\(^{2+}\) near the periphery, but, in the core, mitochondria may be forced to accumulate excessive Ca\(^{2+}\), destroying themselves in the process. The degeneration of mitochondria could lead to diminished ATP production and to the degeneration of a central, possibly compartmented, core. The disorganization of the central core might be brought about by higher core levels of Ca\(^{2+}\), which could cause contraction at the core, but not at the periphery, and lead to myofibrillar streaming and membrane disorganization. Elevated Ca\(^{2+}\) may stimulate the transcription of genes that encode proteins of the sarcoplasmic reticulum and transverse tubules that would be required to reestablish Ca\(^{2+}\) homeostasis. This may stimulate proliferation of internal membrane proteins at the transcriptional level. The phenotypic effects would be the formation of a disorganized, metabolically deficient core, which could lead to cell death and muscle atrophy. (Adapted from MacLennan, D. H. and Phillips, M. S. (1995) In Ion Channels and Genetic Diseases (Dawson, D. C. and Frizzell, R. A., eds.). Rockefeller University Press, New York, pp. 89–100, with permission from Rockefeller University Press.)
Pumps and exchangers in the plasma membrane might be more effective in protecting the periphery of the cell than the interior of the cell where the full burden of regulation of excess \( \text{Ca}^{2+} \) would fall on the sarcoplasmic reticulum and mitochondria. Mitochondria, which have a high capacity for \( \text{Ca}^{2+} \) uptake, would, undoubtedly, participate in removal of excess \( \text{Ca}^{2+} \) from the central core of the muscle cell and might destroy themselves in an effort to protect the cell from \( \text{Ca}^{2+} \)-induced necrosis (Wroge and Pena, 1976). Loss of mitochondria from the center of the cell would, in turn, lead to lower ATP synthesis and might be an underlying cause of the disorganization of the central core, leading to muscle weakness and muscle atrophy. Elevated \( \text{Ca}^{2+} \) in the interior of the muscle cell might have the same effects on the core of the muscle cell as MH would have on the whole cell. Of most interest would be its effects on muscle contraction. The differential contraction of the core of the muscle, in relation to the periphery, could lead to the disorganization and “streaming” of both fibers and membrane systems that is observed in the central cores. The profusion of sarcoplasmic reticulum and transverse tubules might be induced at the gene level by high local \( \text{Ca}^{2+} \) concentrations. Thus, mutations in \( \text{RYR1} \) can lead to a spectrum of pathophysiological responses that range from muscle hypertrophy to muscle atrophy.

When CCD mutations were introduced into full-length rabbit \( \text{Ca}^{2+} \) release channel cDNA and expressed transiently in HEK-293 cells, resting \( \text{Ca}^{2+} \) concentrations were higher than in cells expressing wild-type or MH mutant \( \text{RYR1} \) proteins, suggesting that the CCD mutants are exceptionally permeable (Tong et al., 1999). Under these conditions, HEK-293 cells expressing both MH and CCD mutant \( \text{RYR1} \) exhibited lower maximal peak amplitudes of caffeine-induced \( \text{Ca}^{2+} \) release than cells expressing wild-type \( \text{RYR1} \), suggesting that both MH and CCD mutants were more leaky so that \( \text{Ca}^{2+} \) stores were reduced in size. The content of endogenous sarco(end)oplasmic reticulum \( \text{Ca}^{2+} \) ATPase isofrom 2b (SERCA2B) was increased in HEK-293 cells expressing wild-type or mutant \( \text{RYR1} \), supporting the view that sarco- or endoplasmic reticulum \( \text{Ca}^{2+} \) storage capacity is increased as a compensatory response to an enhanced \( \text{Ca}^{2+} \) leak.

**Brody Disease**

Brody disease (Brody, 1969) was first defined as a disorder of muscle function characterized by painless muscle cramping and exercise-induced impairment of muscle relaxation. In a normal muscle contraction/relaxation cycle, \( \text{Ca}^{2+} \) is released from the sarcoplasmic reticulum into the cytoplasm, where it binds to troponin in the thin filament, releasing constraints on the interaction between actin and myosin and inducing muscle contraction (Zot and Potter, 1987). Then, \( \text{Ca}^{2+} \) is pumped back into the lumen of the sarcoplasmic reticulum by a \( \text{Ca}^{2+} \) pump to initiate relaxation. In his studies of a skeletal muscle biopsy, Brody (1969) showed that the sarcoplasmic reticulum from his patient was deficient in both \( \text{Ca}^{2+} \) uptake and \( \text{Ca}^{2+} \)-ATPase activity. Consequently, his report focused attention on the possibility that defects in the \( \text{Ca}^{2+} \) pump might underlie the disease. This possibility was supported by studies in which fast-twitch skeletal muscle fibers from four Brody patients were shown to be deficient in \( \text{Ca}^{2+} \)-ATPase (Karp et al., 1986; Danon et al., 1998), but it was undermined by studies of Benders et al. (1994) in which a 50% decrease in the activity of the fast-twitch \( \text{Ca}^{2+} \)-ATPase isofrom, SERCA1, was recorded, even though no reduction in protein content was observed. Recent analysis of the \( \text{ATP2A1} \) gene that encodes SERCA1 has also provided conflicting results. In two Brody families, both \( \text{ATP2A1} \) alleles were shown to code for truncated, inactive forms of SERCA1 (Odermatt et al., 1996), but, in four other Brody families, \( \text{ATP2A1} \) was unaltered (Zhang et al., 1995; Odermatt et al., 1996). Thus, current research is aimed at the understanding of the heterogeneous genetic origins of Brody disease (or syndrome).

The diagnosis of Brody disease (Odermatt et al., 1996) is based on a lifelong history of difficulty in performing sustained, strenuous muscular activities, such as running upstairs, because the muscles stiffen during exercise and, temporarily, cannot be used. The exercise-induced delay in muscle relaxation involves the legs, arms, and eyelids, and may be worse in cold weather. Clinical examination demonstrates normal strength with a single effort, normal sensation, and normal deep tendon reflexes, but progressive difficulty in relaxing muscles during repeated forceful contraction. Percussion myotonia is absent. Standard needle electromyography shows normal spontaneous, insertion, and voluntary activity, but no electrical activity nor myotonic discharges in the muscles after exercise and during the delayed relaxation. The disease is not life threatening and no specific therapy is indicated.

There are no recorded estimates of the incidence of Brody disease, partly because the syndrome is difficult to diagnose and partly because of its rarity. Inheritance of Brody disease is autosomal recessive in the two cases where a genetic diagnosis has been possible (Odermatt et al., 1996), but it is also heterogeneous in origin (Zhang et al., 1995) and, in some families, inheritance might be dominant. A rough estimate of the incidence of the disease might be 1 in 10,000,000 births. While this figure seems rare.
Indeed, the incidence of carriers required to achieve this incidence would be 1 in 1600, if the disease were invariably autosomal recessive.

Physiological Basis for Brody Disease

All of those who have investigated the role of the Ca\textsuperscript{2+}-ATPase in Brody disease have found diminished Ca\textsuperscript{2+}-ATPase activity, but reports of the fraction of Ca\textsuperscript{2+}-ATPase that remains have varied dramatically (Karpati et al., 1986; Danon et al., 1988; Taylor et al., 1988; Benders et al., 1994). Brody (1969) reported that ATP-dependent Ca\textsuperscript{2+} uptake in the microsomal fraction of skeletal muscle from his patient was reduced to 25% of control values. Studies by Taylor et al. (1988) showed that the Ca\textsuperscript{2+}-ATPase activity in skeletal muscle microsomes of a single Brody patient was reduced to about 10% of the activity of control samples. Karpati et al. (1986) measured Ca\textsuperscript{2+} uptake in microsomal fractions from Brody muscle and found it to be reduced to 2% or less of control values.

In mammalian tissues, three different ATP2A genes encode five different sarco(endo)plasmic reticular Ca\textsuperscript{2+}-ATPase (SERCA) proteins (MacLennan et al., 1983; Brandl et al., 1986, 1987; Lytton and MacLennan, 1988; Burk et al., 1989; Lytton et al., 1989). The SERCA1a protein is encoded by the ATP2A1 gene located on human chromosome 16p12.1–p12.2 (Callen et al., 1995), the ATP2A2 gene that encodes SERCA2a is located on chromosome 12q23–q24, and the ATP2A3 gene that encodes SERCA3 is located on chromosome 17q13.3 (Dode et al., 1996). The human ATP2A1 gene contains 23 exons, spanning about 26 kb of genomic DNA (Zhang et al., 1995). Exon 22 is retained to form the adult SERCA1a protein, ending in Gly, while alternative splicing, leading to the removal of exon 22, leads to the formation of the neonatal SERCA1b protein, terminating in the sequence Glu-Asp-Pro-Glu-Asp-Glu-Arg-Arg-Lys (Brandl et al., 1986, 1987). The SERCA1a protein accounts for more than 99% of SERCA isoforms expressed in adult fast-twitch skeletal muscle (type II) fibers, while SERCA1b predominates in neonatal fibers (Brandl et al., 1987; Wu et al., 1995). A similar, but not identical, splicing occurs in the ATP2A2 gene, creating the cardiac/slow-twitch isoform SERCA2a and the smooth muscle/nonmuscle isoform, SERCA2b (Lytton and MacLennan, 1988). The SERCA1 isoforms are not expressed to any significant extent in any tissue other than fast-twitch skeletal muscle, while SERCA2 isoforms are expressed to some extent in virtually all other tissues (Wu et al., 1995). SERCA3 also has complex splicing at the C-terminal end (Poch et al., 1998).

Supporting evidence for the view that defects in the Ca\textsuperscript{2+}-ATPase are involved in Brody disease was presented by Karpati et al. (1986) and by Danon et al. (1988), who showed that polyclonal and monoclonal antibodies against the Ca\textsuperscript{2+}-ATPase from chicken fast-twitch skeletal muscle sarco/endoplasmic reticulum reacted only poorly, if at all, with histochecmmical type 2 (fast-twitch) fibers in sections of the skeletal muscle of four Brody patients. The antibodies did, however, react with the Ca\textsuperscript{2+}-ATPase in type 1 fibers. These studies suggested that the Ca\textsuperscript{2+}-ATPase protein was either absent from type 2 fibers or was present in a form with altered antigenicity. In cross sections of muscle biopsies, type 2 fibers appeared angular and atrophied, whereas type 1 fibers appeared normal. Since the SERCA1 isoform predominates in type 2 (fast-twitch) fibers, while the SERCA2 isoform predominates in type 1 (slow-twitch) fibers (Brandl et al., 1987), the studies of Karpati et al. (1986) and of Danon et al. (1988) imply that defects in the SERCA1 isoform of the Ca\textsuperscript{2+}-ATPase might be directly involved in the manifestation of Brody disease.

Benders et al. (1994) analyzed both Ca\textsuperscript{2+}-ATPase activity and Ca\textsuperscript{2+}-ATPase protein content in 10 Brody patients. Using an antibody specific against SERCA1, they estimated that 83% of the total Ca\textsuperscript{2+}-ATPase in both Brody patient and control muscle homogenates was SERCA1. Moreover, the content of total Ca\textsuperscript{2+}-ATPase protein and SERCA1 protein, measured both by immunoreactivity and by phosphorylation, was identical in the two sample populations. The Ca\textsuperscript{2+}-stimulated ATPase activity in Brody muscle homogenates, however, was reduced to only 50% of the activity found in comparable samples from normal patients. A similar pattern was observed when ATPase activities and protein contents were measured in cells cultured from normal and Brody disease muscle.

The findings of Karpati et al. (1986) and those of Benders et al. (1994) implicate SERCA1 in Brody disease, but support very different views of its etiology. The results of Karpati et al. (1986) are consistent with mutations in ATP2A1 that cause virtually 100% loss of SERCA1 protein and Ca\textsuperscript{2+}-ATPase activity. Such mutations might affect translation or they might result in a protein that is degraded before it is functionally incorporated into the bilayer. The studies of Benders et al. (1994) are consistent with mutations in ATP2A1 that do not affect SERCA1 expression, but diminish its V\textsubscript{max} to about 50% of control values. Studies of mutagenesis of the Ca\textsuperscript{2+}-ATPase (MacLennan et al., 1992) demonstrate many examples of mutations or small deletions that affect expression and stability of the Ca\textsuperscript{2+}-ATPase and many examples of mutations that reduce Ca\textsuperscript{2+}-ATPase activity by 10–90%.
Genetic Analysis of Brody Disease

In order to determine whether genetic defects exist in the ATP2A1 gene, sequencing of ATP2A1 cDNA or genomic DNA has now been carried out on six unrelated Brody disease probands (Zhang et al., 1995; Odermatt et al., 1996, 1997). Amplification of the full-length ATP2A1 cDNA from two of these patients did not reveal any deletion or apparent alternative splicing, nor were any defects detected in the analysis of the ATP2A1 cDNAs from these two patients (Zhang et al., 1995). Although quantitative analysis of mRNA levels was not carried out, it was evident from the ease with which PCR amplification was accomplished that the mRNA was relatively abundant. In a third Brody proband, the 23 individual ATP2A1 exons, plus the flanking intron sequence and several hundred base pairs of upstream sequence in the ATP2A1 gene, were amplified and sequenced. Again, no evidence for a mutation in ATP2A1 was found (Zhang et al., 1995).

In a study of two more Brody disease families, clinical evaluation identified three individuals in one family and two individuals in a second family with typical signs of Brody disease. A 50% reduction in Ca\textsuperscript{2+}-ATPase and Ca\textsuperscript{2+}-transport activities had been reported in the first family (Benders et al., 1994), while a complete loss in SERCA1 had been reported in the second family (Karpati et al., 1986). Since the ATP2A1 gene that encodes SERCA1 has been localized to chromosome 16 in the interval between D16S297 and D16S288 (Callen et al., 1995), haplotype analysis was carried out on these families using genetic markers between D16S295 and D16S304 (Odermatt et al., 1996). In the first family, both parents inherited the same haplotype for the 6.6 cM interval between markers D16S288 and D16S304. Two affected patients (one affected family member was unavailable) inherited this common haplotype from each parent, but a crossover between markers D16S298 and D16S300 in the paternal chromosome inherited by one of them limited the region of haplotype identity so that the two were homozygous only for the D16S288/D16S298 haplotype. The unaffected parents and an unaffected sibling were heterozygous for this haplotype and two other unaffected siblings did not carry this haplotype in either chromosome. These results were consistent with autosomal recessive inheritance of Brody disease.

In the second family, the parents also shared an identical haplotype on most of one chromosome. Both affected patients inherited the maternal copy of this common haplotype and both inherited the different paternal haplotype. Inheritance by the two patients of the same two ATP2A1 intervals was, again, consistent with the autosomal recessive inheritance of Brody disease, suggesting that, for these two families, a defect was located within the interval flanked by D16S297 on the telomeric side and by D16S300 on the centromeric side (containing the ATP2A1 gene).

Sequencing of exon 7 in the proband from the first family and comparison with normal ATP2A1 sequence (Zhang et al., 1995) revealed the homozygous mutation of C592 to T, resulting in the mutation of the Arg\textsuperscript{198} codon, CGA, to the stop codon, TGA (Table 27.1). The truncated product would be devoid of phosphorylation, nucleotide-binding, and Ca\textsuperscript{2+}-binding domains and, almost certainly, of activity (MacLennan et al., 1992). Genetic data were fully consistent with the recessive inheritance of the C592 to T mutation as the causal factor for Brody disease in the first family.

Sequencing of amplified genomic DNA from the proband in the second family revealed two different mutations in ATP2A1, consistent with the inheritance of the two different haplotypes. The mutation of C2023 to A in the TGC codon for Cys\textsuperscript{673} in exon 15 of the paternally inherited chromosome created the stop codon, TGA, predicted to lead to a truncated protein of 674 amino acids (Table 27.1). The truncated gene product would contain phosphorylation and nucleotide-binding domains, but the Ca\textsuperscript{2+}-binding domain would be disrupted (MacLennan et al., 1992). The mutation of the invariant GT dinucleotide to CT at the splice donor site of intron 3 in the maternally inherited chromosome (Table 27.1) was predicted to lead preferentially to skipping of exon 3 and less frequently to partial retention of intron 3 (Krawczak et al., 1992). If exon 2 of ATP2A1 was spliced to exon 4 and transcribed, the product would be truncated, consisting of 45 normal amino acids, followed by 5 novel amino acids. If intron 3 were retained partially and transcribed, the product would also be truncated, consisting of 73 normal amino acids, followed by 49 novel amino acids. Both potential gene products would be missing phosphorylation, nucleotide-binding and Ca\textsuperscript{2+}-binding domains, and activity (MacLennan et al., 1992). Thus, in the second family, the inheritance of Brody disease was autosomal recessive and was associated with compound heterozygosity for the two ATP2A1 mutations, leading to the prediction of total loss of SERCA1 protein and activity.

Since one of the patients in the original study of Zhang et al. (1995) had been included in the immunohistochemical analysis of Karpati et al. (1986) and had been scored as SERCA1-deficient, the sequence of the ATP2A1 gene in this family was re-evaluated (Odermatt et al., 1997). Two affected brothers in this family were found to have the identical haplotype in the segment of chromosome 16p12 where ATP2A1 is located. Resequencing of the ATP2A1 gene then led to the discovery of the homozygous deletion of a C in
a series of three CAs in exon 5, leading to a frameshift at Pro147 in both affected brothers that would truncate and inactivate both copies of SERCA1. Analysis of the ATP2A1 sequence in a sixth Brody family did not uncover any defect (Odermatt et al., 1997).

The three families in which ATP2A1 mutations truncate and inactivate SERCA1 demonstrate recessive inheritance of Brody disease. For those families in which Brody disease shows dominant inheritance, ATP2A1 is probably not the causal gene (Odermatt et al., 1997). It is, however, conceivable that defects might occur in a gene that encodes a protein which either directly or indirectly modifies the specific activity of SERCA1. In a study of the sarcoplasmic (SLN) gene, which encodes a potential SERCA1 inhibitor, Odermatt et al. (1997) were unable to detect mutations that might be linked to Brody disease. Wevers et al. (1992) and Bender et al. (1994) showed that dantrolene, which reduces myofibrillar Ca2+ concentration by blocking Ca2+ release from the sarcoplasmic reticulum, had no effect on the contracture in the dantrolene-resistant myopathy.
mic reticulum (Ohta et al., 1990; Nelson and Lin, 1993), is effective in the treatment of some, but not all, Brody patients. Therefore, the possibility must be considered that mutations in other components of muscle Ca\(^{2+}\) regulation might be responsible for the clinical manifestation of some forms of Brody disease.

In spite of the predicted absence of SERCA1 in the several Brody patients who inherited ATP2A1 gene defects, all are able to relax their fast-twitch skeletal muscles, although at a significantly reduced rate. In cultured muscle cells from both patients in one of these families and controls, the sarcoplasmic Ca\(^{2+}\) concentration at rest and the increase in intracellular Ca\(^{2+}\) concentration after addition of acetylcholine were found to be the same (Benders et al., 1994). However, the time required to reach resting intracellular Ca\(^{2+}\) levels after Ca\(^{2+}\) release was increased several-fold in cells derived from these patients. Thus, the phenotype was consistent with reduced SERCA1 activity, but not with complete loss of SERCA1 function.

Concentrations of Ca\(^{2+}\) in Brody muscle might be lowered through a combination of other mechanisms which regulate Ca\(^{2+}\) in muscle (Carafoli, 1987). There might be compensatory Ca\(^{2+}\) removal by plasma membrane Ca\(^{2+}\)-ATPases (PMCA), by Na\(^+\)/Ca\(^{2+}\) exchangers in the plasma membrane, by mitochondrial Ca\(^{2+}\) uptake, or by the proliferation of sarcoplasmic or endoplasmic reticulum that contains compensating levels of SERCA2 (MacLennan et al., 1985) or SERCA3 (Burk et al., 1989) isoforms (Fig. 3). Of these possible compensatory processes, only the latter would be predicted to result in Ca\(^{2+}\) loading of the sarcoplasmic reticulum, a process necessary for subsequent muscle contraction. As an alternative, refilling of Ca\(^{2+}\)-depleted sarcoplasmic reticulum might be possible through some form of capacitative Ca\(^{2+}\) entry (Berridge, 1995).

The literature on the content of Ca\(^{2+}\)-ATPase protein in Brody patients is conflicting. Benders et al. (1994) reported a normal Ca\(^{2+}\)-ATPase protein content and a 50% reduction in Ca\(^{2+}\)-ATPase activity in two patients from one ATP2A1-deficient family, while genetic study would predict a complete loss of SERCA1 protein and function (Odermatt et al., 1996). Further evaluation of these discrepancies will be necessary. Present findings, however, provide clear genetic evidence that ATP2A1 is a candidate gene for at least one autosomal recessive form of Brody disease and support the earlier view that the loss of SERCA1 function underlies the manifestation of this form of the disease. Further investigation will also be necessary to determine the cause of the identical syndrome in patients who do not have an ATP2A1 defect and who seem to inherit an autosomal dominant form of the disease (Odermatt et al., 1997).

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PATHOLOGY OF CALCIUM-TRANSFERRING MEMBRANE SYSTEMS 627


