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TRANSLATIONAL PHYSIOLOGY

Increased sensitivity of the ryanodine receptor to halothane-induced oligomerization in malignant hyperthermia-susceptible human skeletal muscle

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Glover, Louise, James J. A. Heffron, and Kay Ohlendieck. Increased sensitivity of the ryanodine receptor to halothane-induced oligomerization in malignant hyperthermia-susceptible human skeletal muscle. *J Appl Physiol* 96: 11–18, 2004. First published September 5, 2003; 10.1152/japplphysiol.00537.2003.—Mutations in the skeletal muscle RyR1 isoform of the ryanodine receptor (RyR) Ca^{2+}-release channel confer susceptibility to malignant hyperthermia, which may be triggered by inhalational anesthetics such as halothane. Using immunoblotting, we show here that the ryanodine receptor, calmodulin, junctin, calsequestrin, sarcalumenin, calreticulin, annexin-VI, sarco(endo)plasmic reticulum Ca^{2+}-ATPase, and the dihydropyridine receptor exhibit no major changes in their expression level between normal human skeletal muscle and biopsies from individuals susceptible to malignant hyperthermia. In contrast, protein gel-shift studies with halothane-treated sarcoplasmic reticulum vesicles from normal and susceptible specimens showed a clear difference. Although the α_2-dihydropyridine receptor and calsequestrin were not affected, clustering of the Ca^{2+}-ATPase was induced at comparable halothane concentrations. In the concentration range of 0.014–0.35 mM halothane, anesthetic-induced oligomerization of the RyR1 complex was observed at a lower threshold concentration in the sarcoplasmic reticulum from patients with malignant hyperthermia. Thus the previously described decreased Ca^{2+}-loading ability of the sarcoplasmic reticulum from susceptible muscle fibers is probably not due to a modified expression of Ca^{2+}-handling elements, but more likely a feature of altered quaternary receptor structure or modified functional dynamics within the Ca^{2+}-regulatory apparatus. Possibly increased RyR1 complex formation, in conjunction with decreased Ca^{2+} uptake, is of central importance to the development of a metabolic crisis in malignant hyperthermia.

calcium homeostasis; excitation-contraction coupling; sarcoplasmic reticulum; supramolecular complex; triad

ION CHANNELOPATHIES EXHIBIT recurrent patterns of mutations and have common clinical features. Primary abnormalities in Na^{+}, K^{+}, Ca^{2+}, and Cl^{-} channels may lead to hypoexcitability resulting in periodic paralysis, hyperexcitability causing myotonia, or susceptibility to malignant hyperthermia (MH) (23). The pharmacogenetic disorder malignant hyperthermia is a potentially fatal, inherited myopathy, characterized by a fulminant metabolic response to volatile anesthetic agents in predisposed individuals (33, 39). The symptoms of a hypermetabolic crisis, such as hypoxia, acidosis, and hyperthermia, are widely attributed to a defect of intracellular Ca^{2+} homeostasis (31, 37). Changes in the sensitivity of the sarcoplasmic reticulum (SR) Ca^{2+}-release mechanism to various activating ligands, including halogenated anesthetics and 4-chloro-*m*-cresol containing preparations of depolarizing muscle relaxants, have been reported and characterized in susceptible skeletal muscle (21, 36, 43, 45, 49). Such changes are largely thought to represent the phenotypic manifestation of point mutations in the major MH-susceptible (MHS) locus, i.e., the gene encoding the human skeletal muscle ryanodine receptor (RyR) 1 (RyR1) Ca^{2+} release channel (2). Over 20 mutations therein have been defined (24, 33), accounting for susceptibility to MH in over 50% of affected pedigrees (2). Furthermore, heterologous expression of mutated ryanodine receptors in human embryonic kidney cells has been shown to confer an enhanced sensitivity of Ca^{2+} release in response to halothane compared with wild types (47). This strongly suggests that these mutations alone are sufficient to produce susceptibility to this muscular disorder, without the requirement for any additional muscle-specific conditions (35).

Although Ca^{2+} release from the SR in response to anesthetics, such as halothane, is the generally accepted molecular pathogenesis underlying an episode of MH (37), the exact mechanism by which an anesthetic agent physically elicits increased RyR1 channel opening is not well understood. An intimate understanding of the molecular mechanism of general anesthesia has preceded the true elucidation of such a process, because the acceptance of proteins as primary molecular targets for volatile agents is a relatively recent advancement (12). The traditional model of anesthetic action, a nonspecific perturbation of the phospholipid bilayer of neuronal membranes (22, 29), has been largely discarded in favor of direct anesthetic binding to protein targets (16). The major hypotheses of the mechanism of general anesthesia suggest a variety of different molecular scenarios (13), compounded by the enormous structural variations between agents displaying anesthetic potency. Although no defined protein binding site has yet been identified, the predicted targets of these agents are hydrophobic sites exhibiting some polar character, whose features are sufficiently general to be widespread (13); primary candidates include the hydrophobic internal cavity regions of ligand-gated ion chan-

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Table 1. *IVCT data of MHS and MHN biopsied muscle*

Patient Number	Amplitude of Halothane Contracture at 0.54 mM, mN	Amplitude of Caffeine Contracture at 2 mM, mN
MHS 1	5	2
MHS 2	16	3
MHS 3	23	8
MHN 1–3	0.0	0.0

Muscle was deemed as malignant hyperthermia susceptible (MHS) when 2% (vol/vol) halothane or 2 mM caffeine generated 2 mN force (40). Each of the 3 normal (MHN) samples generated no detectable tension at the respective halothane and caffeine concentrations.

nels, whereby drug binding triggers anesthesia via potentiation of postsynaptic inhibitory channel activity (16).

A number of observations have lent credibility to the idea of proteins as anesthetic molecular targets. Volatile agents have been shown to interact with model proteins such as bovine serum albumin (11) and the firefly enzyme luciferase (16); both examples are lipid-free, soluble proteins. The anomalous lack of anesthetic potency of long-chain compounds (1) is most simply described by anesthetics binding to protein pockets or clefts with circumscribed dimensions (14). However, perhaps the most compelling evidence for direct protein binding comes from observations of stereoselectivity (15). Even the relatively simple agent isoflurane displays stereoselective effects on neuronal ion channels, a feature that does not extend to pure lipid bilayers (15). What remains unresolved is the question of why some proteins are highly sensitive to anesthetics whereas others are not, and the nature of those protein binding sites that define sensitivity. In addition, the structural and dynamic consequences of anesthetic binding are relatively unknown and difficult to demonstrate, given that the means to define protein structure is in its relative infancy. Nevertheless, the independent observations that inhalational anesthetics can bind to proteins and then alter protein activity strongly indicate that a change in protein conformation or dynamics must be occurring (13).

To further characterize the SR from muscles of individuals susceptible to MH and determine the conformational or dynamic consequences of halothane binding in normal vs. susceptible fibers, we have analyzed potential changes in the abundance of key Ca^{2+}-regulatory proteins and investigated halothane-induced effects on the quaternary structure of the ryanodine receptor Ca^{2+} release channel. Many studies on human muscle pathophysiology have focused on the vastus lateralis (48), making this particular muscle an excellent candidate for comparative research. We therefore prepared microsomal membranes from normal and MHS vastus lateralis specimens and performed a comprehensive immunoblot analysis. Because the ryanodine receptor is closely associated with various triad proteins (19, 24, 28, 30), we have investigated the relative abundance of accessory marker components located in the cytoplasm (calmodulin, RyR1-associated FK506 binding protein FKBP12), the lumen of the SR [calsequestrin (CSQ), annexin VI, calreticulin, sarcalumenin], the SR membrane (junctin), and the junctional transverse tubules (dihydropyridine receptor).

MATERIALS AND METHODS

Materials. Halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane) was purchased from Sigma Chemical (Poole, Dorset, UK). Protease inhibitors and peroxidase-conjugated secondary antibodies were obtained from Chemicon International (Temecula, CA). Acrylamide stock solutions were from National Diagnostics (Hessle Hull, UK), and chemiluminescence substrates were purchased from Perbio Science UK (Tattenhall, Cheshire, UK). Immobilin NC nitrocellulose membranes were from Millipore (Bedford, MA). All other chemicals were of analytical grade and purchased from Sigma Chemical.

Antibodies. Primary antibodies were purchased from Affinity Bioreagents, Golden, CO [monoclonal antibodies $VIIID1_2$ to fast CSQ, VE121G9 to the fast sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) 1 isoform of the Ca^{2+}-ATPase, IID8 to the slow SERCA2 isoform of the Ca^{2+}-ATPase, XIIC4 to sarcalumenin, 1A to the α_{1S}-subunit of the dihydropyridine receptor, 20A to the α_2-subunit of the dihydropyridine receptor, 6D4 to calmodulin, and polyclonal antibodies PA1-026 to FKBP12, and PA3-900 to calreticulin]; Upstate Biotechnology, Lake Placid, NY (polyclonal antibody to the RyR1 isoform of the ryanodine receptor Ca^{2+}-release channel); Transduction Laboratories, Lexington, KY (monoclonal antibodies to annexin II and annexin VI); Chemicon International (monoclonal antibody to glyceraldehyde-phosphate dehydrogenase); and Sigma Chemical (monoclonal antibodies MY-32 to the fast isoform and NOQ7.5.4D to the slow isoform of the heavy meromyosin portion of myosin). A polyclonal antibody raised against junctin was generously donated by Dr. Steven Cala (Wayne State University School of Medicine, Detroit, MI).

Isolation of microsomal membranes. Before membrane preparation, in vitro contracture testing (IVCT) of biopsied muscle for susceptibility to MH was performed according to the European MH group protocol (40). The microsomal fraction enriched in vesicles derived from the SR was obtained from three MHS and three MH-normal (MHN) biopsies after dissection of muscle fibers required for IVCT. This was carried out with the express permission of each patient and approved by the Ethics Committee of the Medical School

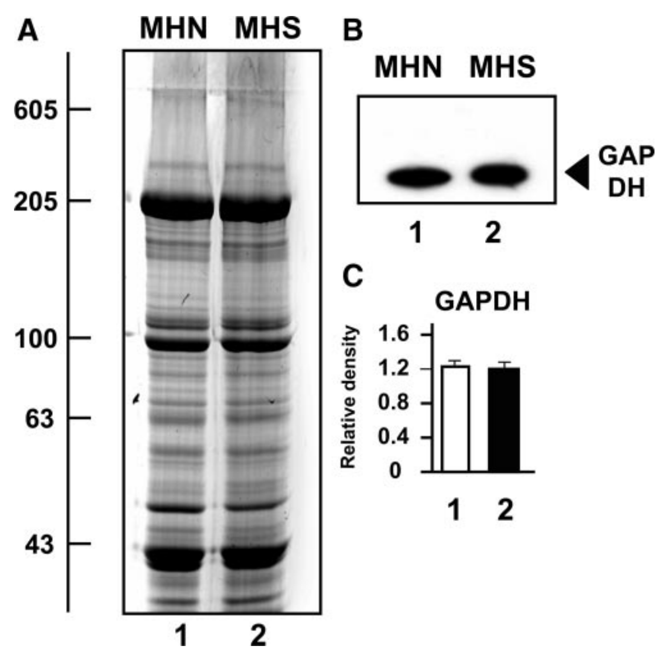

Fig. 1. Characterization of microsomes from malignant hyperthermia (MH)-susceptible (MHS) human muscle. *A*: silver-stained gel. *B*: immunoblot labeled with an antibody to glyceraldehyde phosphate dehydrogenase (GAPDH). *C*: graphical presentation of the densitometric analysis of enhanced chemiluminescence (ECL)-stained blots ($n = 3$). There is no significant difference in the expression of GAPDH in MH-normal (MHN) vs. MHS samples. *Lanes 1* and *2* represent microsomal muscle membranes derived from MHN and MHS individuals, respectively. Immunodecorated protein bands are marked by an arrowhead, and molecular mass standards ($\times 10^{-3}$) are indicated on the left.

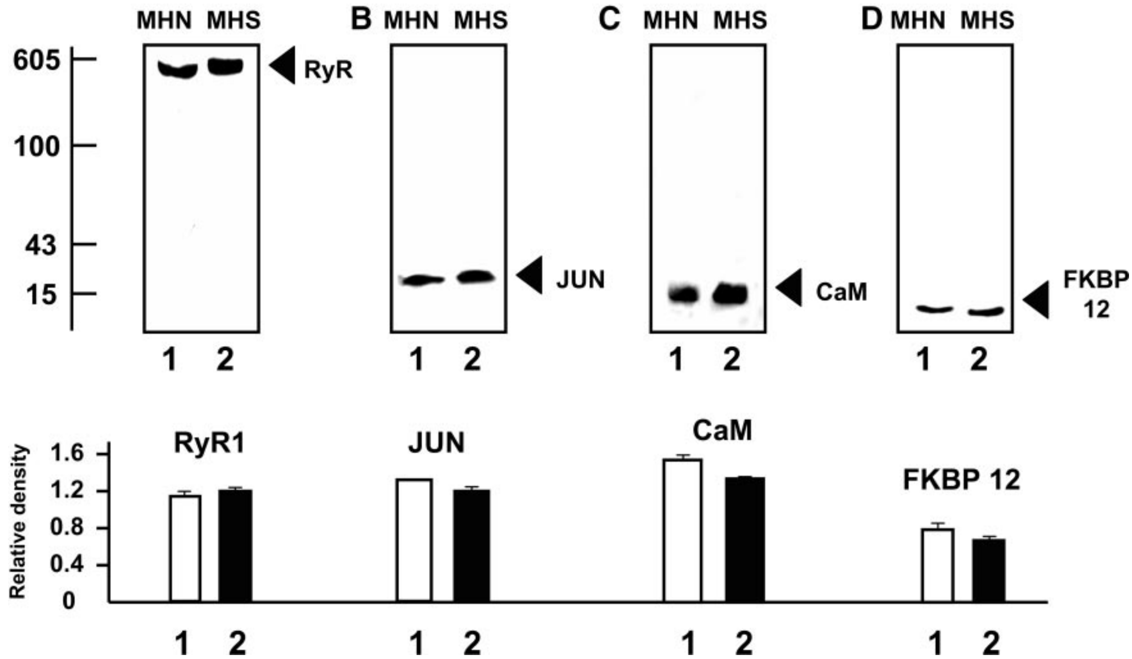

Fig. 2. Immunoblot analysis of the ryanodine receptor (RyR) in microsomes from MHS human muscle. *Top*: immunoblots labeled with antibodies to the RyR (*A*), junctin (JUN) (*B*), calmodulin (CaM) (*C*), and the FK506 binding protein (FKBP12) (*D*). *Bottom*: graphical presentation of the densitometric analysis of ECL-stained blots ($n = 3$). There is no significant difference in the expression of the analyzed proteins in MHN vs. MHS samples. *Lanes 1* and *2* represent microsomal muscle membranes derived from MHN and MHS individuals, respectively. Immunodecorated protein bands are marked by arrowheads, and molecular mass standards ($\times 10^{-3}$) are indicated on the left.

of University College Cork. Preparation of the heavy SR fraction from human vastus lateralis muscle was carried out as previously described (42). All procedures were carried out on ice at 0–4°C, and all isolation buffers contained 5 μg/ml of aprotonin, 5 μg/ml of leupeptin, and 5 μg/ml of pepstatin. The isolated membrane fraction was resuspended at a final protein concentration of 10 mg/ml and stored at −70°C for use within 1 mo of preparation. Ca^{2+}-ATPase activity was measured as previously described (10), and protein concentration was determined according to the method of Bradford (5) with the use of bovine serum albumin as a standard.

Incubation of muscle membranes with halothane. SR vesicles, resuspended in 10 mM Tris·Cl, pH 7.0, 5 mM EGTA, 0.1 M NaCl, 0.3 M sucrose, were incubated for 30 min with 0.014–1.375 mM halothane at 4°C. Halothane concentrations were based on previously published procedures (27) estimating clinical levels of this drug to be ~0.03–0.05 mol halothane per mole of lipid in the bilayer (46). Halothane was added from an acetone-containing 50% (vol/vol) stock solution. To detect halothane-induced complex formation, proteins were treated with a nondenaturing buffer system before electrophoretic separation (17).

Gel electrophoresis and immunoblot analysis. Polyacrylamide gel electrophoresis was performed under native, semi-native, or denaturing conditions, as previously described in detail (7, 17). Electrophoretic analyses were carried out by use of 5% (wt/vol) or 6% (wt/vol) resolving gels with a 4% (wt/vol) stacking gel, employing a Mini-Protean III electrophoresis system from Bio-Rad Laboratories (Hemel Hempstead, Herts, UK). Electrophoretic transfer to nitrocellulose sheets for 70 min at 100 V, incubation with primary and peroxidase-conjugated secondary antibodies, as well as immunodecoration and visualization by enhanced chemiluminescence were performed according to established methods (4). Densitometric scanning of enhanced chemiluminescence blots was carried out on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA) with ImageQuant V3.0 software.

RESULTS

After the diagnostic evaluation of biopsied vastus lateralis muscle specimens for susceptibility to MH by IVCT (Table 1), this study focused on the analysis of microsomal membranes derived from MHS muscle (Fig. 1), evaluated potential changes in the expression of ryanodine receptor-associated proteins (Figs. 2 and 3), determined halothane-induced effects on the quaternary structure of protein complexes of the SR (Figs. 4–6), and measured the effect of halothane on Ca^{2+}-ATPase activity. Our immunoblot survey included accessory Ca^{2+}-release channel markers of the cytoplasm, the lumen and membrane of the SR, and the junctional transverse tubules. To determine whether the mutant status of MHS SR is reflected by modified protein complex formation, we performed comparative protein gel-shift analyses with halothane. Because of the relative scarcity of human tissue material available, this study had to be performed with a limited amount of both individual samples and total amounts of available tissue.

Characterization of MHS vastus lateralis muscle. As shown in Table 1, the diagnostic data clearly revealed susceptibility to MH in *patients MHS-1* to *MHS-3*. IVCT was carried out according to the European MH group protocol. Muscle specimens were deemed MHS when 0.54 mM halothane and 2 mM caffeine generated ≥2 mN force (40). Each of the three MHN biopsies generated no detectable tension at the respective caffeine and halothane thresholds. After IVCT analysis, microsomal membranes were prepared from tissue homogenates in the presence of a protease inhibitor cocktail and electrophoretically separated. As illustrated in Fig. 1*A*, the overall protein band pattern is relatively comparable between MHS and MHN

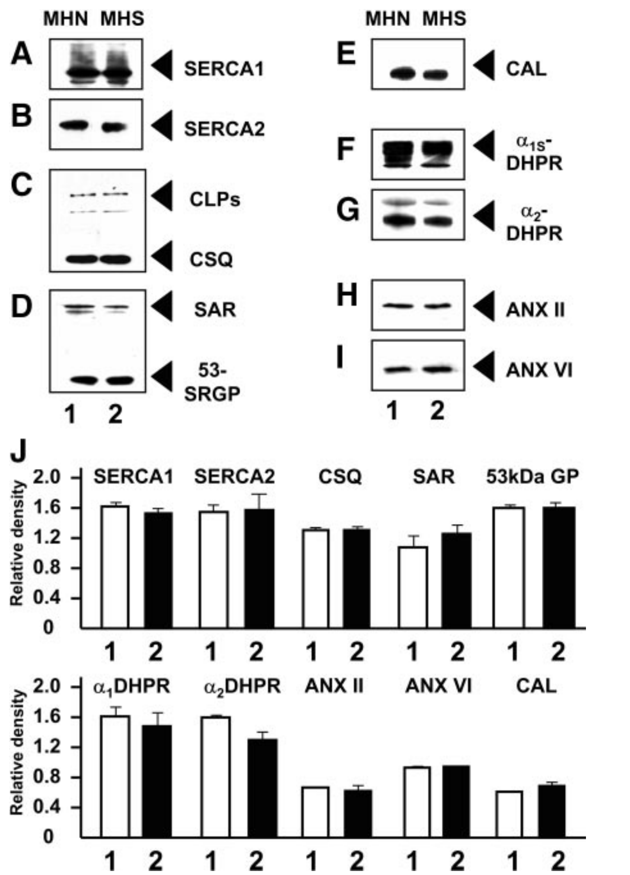

Fig. 3. Immunoblot analysis of key Ca^{2+}-regulatory proteins in microsomes from MHS human muscle. Shown are immunoblots labeled with antibodies to the fast-twitch sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) 1 isoform (*A*) and the slow/cardiac SERCA2 (*B*) isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase, calsequestrin (CSQ) and CSQ-like proteins (CLPs) (*C*), sarcalumenin (SAR), and the SR glycoprotein of 53 kDa (53-SRGP) (*D*), calreticulin (CAL) (*E*), the α_{1S}-subunit (*F*), and the α_2-subunit (*G*) of the dihydropyridine receptor (DHPR), as well as annexin II (ANX II) (*H*) and annexin VI (ANX VI) (*I*). Immunodecorated protein bands are marked by arrowheads. *J*: graphical presentation of the densitometric analysis of ECL-stained blots ($n = 3$). There is no significant difference in the expression of the analyzed proteins in MHN vs. MHS samples. *Lanes 1* and *2* represent microsomal muscle membranes derived from MHN and MHS individuals, respectively.

microsomes. The three main silver-stained clusters at ~40, 100, and 200 kDa represent mostly actin, Ca^{2+}-ATPase, and myosin, respectively. The slow and fast myosin heavy chain (MHC) isoforms, often employed as indicators of general fiber-type distribution in human muscle samples, did not exhibit dramatic differences in their distribution in normal control and MHS specimens (not shown). A key cytosolic marker that appears to form a complex with the triadic Ca^{2+}-release channel is the metabolic enzyme glyceraldehyde 3-phosphate dehydrogenase. The results presented here indicate that the expression of glyceraldehyde 3-phosphate dehydrogenase is not affected in MH (Fig. 1*B*). Its relative expression was unchanged in MHS compared with MHN myofibers (Fig. 1*C*).

Immunoblot analysis of Ca^{2+}-regulatory elements in hyperthermia-susceptible SR. Before our protein gel-shift experiments, we analyzed the expression levels of a number of key elements of Ca^{2+} handling. Figure 2 displays representative results of our immunoblot analysis of electrophoretically separated microsomal proteins and their densitometric evaluation. Both the expression of the RyR1 Ca^{2+}-release channel and the relative density of its putative binding elements was not drastically changed between the two different specimens. Mutations in the polypeptide sequence do not apparently translate to differences in the relative abundance or electrophoretic mobility of the monomeric RyR subunit (Fig. 2*A*). No major changes in the expression of the endogenous regulator junctin (Fig. 2*B*), the RyR1-regulatory protein calmodulin (Fig. 2*C*), and the RyR-associated FK506 binding protein FKBP12 (Fig. 2*D*) were detected between MHN and MHS membrane vesicles.

In analogy to Figs. 1 and 2, the immunoblotting of markers of Ca^{2+} homeostasis did not reveal major differences in the expression of abundant Ca^{2+} pumps, Ca^{2+}-binding proteins, and excitation-contraction coupling elements. The comparable distribution of the fast-twitch SERCA1 isoform (Fig. 3*A*) and the slow/cardiac SERCA2 isoform (Fig. 3*B*) of the SR Ca^{2+}-ATPase between MHN and MHS vesicles agrees with the data on the MHC isoforms. To address this question of whether modified Ca^{2+} cycling through the SR lumen might trigger differences in the expression of ion reservoir elements and their Ca^{2+} storing ability in the MHS SR vs. the MHN SR, electrophoresed membrane fractions were probed for major skeletal muscle and ubiquitous Ca^{2+}-binding elements. No major differences between MHN and MHS SR vesicles were detected in the abundance of the intraluminal protein calsequestrin (CSQ) of the terminal cisternae and its high-molecular-mass isoforms termed CLP (Fig. 3*C*), the longitudinal SR proteins sarcalumenin and 53-SRGP (Fig. 3*D*), or the minor Ca^{2+} storage element calreticulin (Fig. 3*E*). The abundance levels of the main α_{1S}-dihydropyridine receptor and its regulatory α_2-subunit were found not to be drastically different in MHS compared with MHN muscle (Fig. 3, *F* and *G*). To evaluate potential effects of modified Ca^{2+} handling on abundant Ca^{2+}-regulated elements, the widely distributed Ca^{2+}-dependent phospholipid binding proteins of the annexin family were studied. Both annexin II and annexin VI were shown to be not affected in their relative expression in MHS specimens (Fig. 3, *H* and *I*). The densitometric analysis of Ca^{2+}-regulatory elements is summarized in Fig. 3*J*.

Effect of halothane on protein complex formation in hyperthermia-susceptible SR. After treatment with halothane, microsomal proteins were analyzed by use of either native or semi-native gel systems (17). As illustrated in Fig. 4, *A* and *B*, incubation of native SR membranes derived from MHN and MHS myofibers with 0.014–1.375 mM halothane did not induce oligomerization of the native CSQ and CLP protein species, implying that this intraluminal Ca^{2+} binding element probably does not represent a major target for halothane binding. The oligomeric status of a typical triadic membrane protein, the α_2-dihydropyridine receptor, was also not affected by halothane (Fig. 4, *C* and *D*). In contrast, although the vehicle solvent acetone had no effect on the electrophoretic mobility of SERCA1 protein bands separated under semi-native conditions, incubation with halothane reduced the intensity of immunodecoration of the apparent monomer band and clearly caused the appearance of a high-molecular-mass complex in both MHN and MHS membrane vesicles (Fig. 5, *A* and *C*). Concentrations of halothane required to induce SERCA1 complex formation were found to be comparable between normal and MH SR microsomes (Fig. 6*A*).

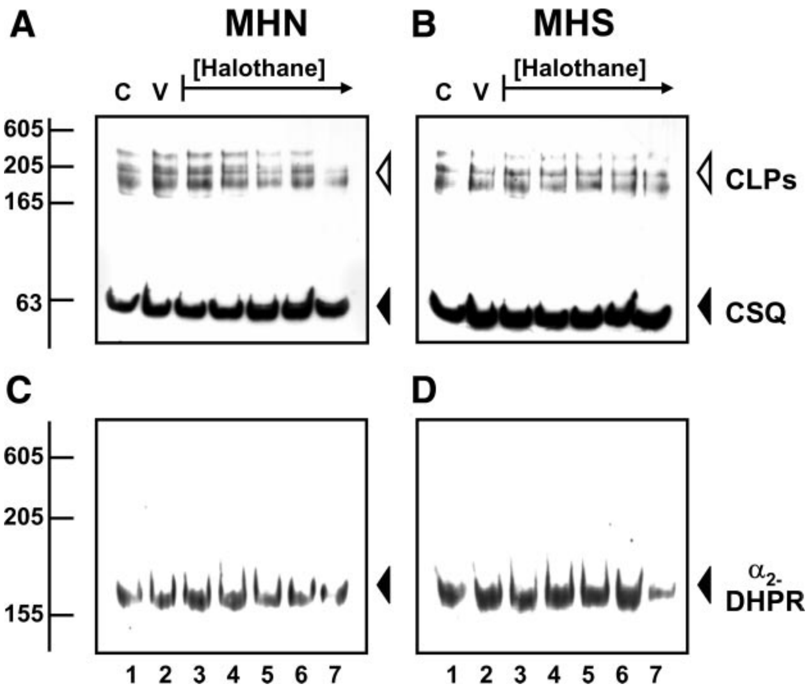

Fig. 4. Effect of halothane on the oligomeric status of CSQ and the α_2-dihydropyridine receptor in microsomes from MHS human muscle. Shown are immunoblots of microsomal membranes from MHN (*A*, *C*) and MHS (*B*, *D*) muscle fibers, labeled with antibodies to CSQ (*A*, *B*) and α_2-DHPR (*C*, *D*). *Lane 1* represents an untreated control (C) sample, *lane 2* is a vehicle (V) control with microsomes incubated with 5% (vol/vol) acetone but no halothane, and *lanes 3-7* represent microsomes incubated with 0.014, 0.07, 0.35, 0.69, and 1.38 mM halothane, respectively. Immunodecorated monomers are indicated by solid arrowheads, and the position of oligomeric structures is marked by open arrowheads. Molecular mass standards ($\times 10^{-3}$) are indicated on the left.

However, halothane-induced oligomerization of the native RyR1 protein species was shown to occur at lower levels of the volatile agent in MHS (Fig. 5*D*) compared with MHN (Fig. 5*B*) SR vesicles. The immunodecoration of a RyR1 containing high-molecular-mass complex in anesthetic-treated MHS vesicles is clearly apparent at a concentration of 0.07 mM halothane, a severalfold lower concentration than that required to induce oligomerization of MHN RyR1 (Fig. 6*B*). Importantly, this oligomerization of RyR1 induced by halothane treatment does not apparently translate to intraluminal CSQ (Fig. 4, *A* and *B*) or the triadic dihydropyridine receptor (Fig. 4, *C* and *D*), suggesting that changes in the quaternary conformation of the Ca^{2+} release channel itself, rather than halothane-mediated interactions with associated proteins, may underlie increased channel opening.

As previous studies have demonstrated a depressed Ca^{2+}-dependent ATPase activity of SERCA isoforms on halothane binding, the Ca^{2+} ATPase activity of MHN and MHS SR

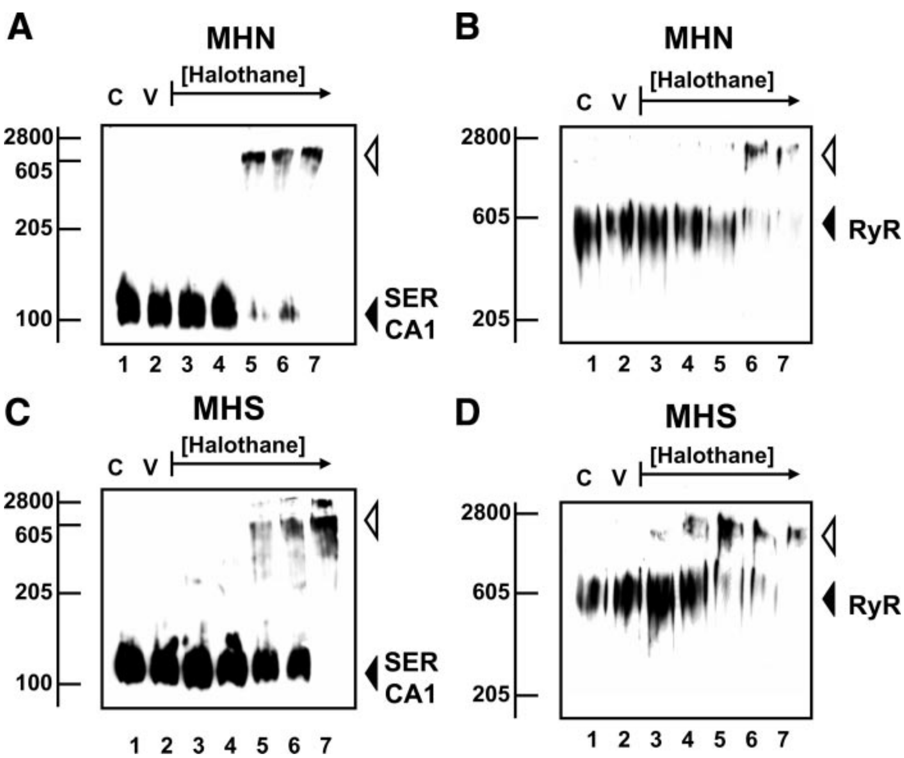

Fig. 5. Effect of halothane on the oligomeric status of the Ca^{2+}-release channel and the Ca^{2+}-ATPase in microsomes from MHS human muscle. Shown are immunoblots of microsomal membranes from MHN (*A*, *B*) and MHS (*C*, *D*) muscle fibers, labeled with antibodies to the fast-twitch SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (*A*, *C*) and the ryanodine receptor Ca^{2+}-release channel isoform RyR1 (*B*, *D*). *Lane 1* represents an untreated control (C) sample, *lane 2* is a vehicle (V) control with microsomes incubated with 5% (vol/vol) acetone but no halothane, and *lanes 3-7* represent microsomes incubated with 0.014, 0.07, 0.35, 0.69, and 1.38 mM halothane, respectively. Immunodecorated monomers are indicated by solid arrowheads, and the position of oligomeric structures is marked by open arrowheads. Molecular mass standards ($\times 10^{-3}$) are indicated on the left.

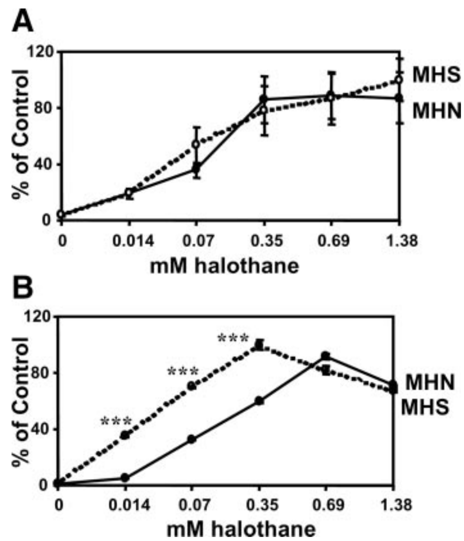

Fig. 6. Halothane-induced oligomerization of the Ca^{2+}-release channel and the Ca^{2+}-ATPase. Shown is the graphical presentation of the densitometric analysis of the effect of halothane on the oligomeric status of the Ca^{2+}-ATPase SERCA1 isoform (*A*) and the Ca^{2+}-release channel RyR (*B*) in microsomes from MHN and MHS human muscle. Statistically very significant differences are represented by $***P < 0.001$. Microsomes were incubated with 0.014, 0.07, 0.35, 0.69, and 1.38 mM halothane. Densitometric scanning of ECL blots ($n = 3$) was performed on a Molecular Dynamics 300S computing densitometer (Sunnyvale, CA) with ImageQuant V3.0 software.

vesicles was measured in the absence of anesthetic, at a low halothane concentration (0.07 mM) and at a 10-fold higher halothane concentration (0.69 mM). The Ca^{2+}-dependent enzyme activity of SR Ca^{2+}-ATPase was measured as the concentration of inorganic phosphate liberated per minute via ATP hydrolysis. SERCA activity was most significantly depressed in MHS SR vesicles, compared with control MHN enzyme activity (not shown), suggesting that a drug-mediated clustering of Ca^{2+} pump units causes an inhibition of enzyme functioning.

DISCUSSION

Ca^{2+} is an important physiological regulator, and because of its relative abundance and complex interactions with various groups of muscle proteins it also plays a central role in the pathophysiology of many muscular disorders (3). In normal skeletal muscle, Ca^{2+} cycling through the various intracellular compartments of fibers is maintained under precise temporal and spatial control. Proper Ca^{2+} handling requires a complex membranous system for the efficient efflux and reuptake of Ca^{2+} ions. Abnormal Ca^{2+} handling leads inevitably to cellular dysfunction. As outlined in recent reviews on the proposed molecular mechanism of MH (24, 33), defective excitation-contraction coupling and impaired Ca^{2+} uptake underlie this muscular disorder. Here, we could show that halothane has a direct effect on the complex formation of the ryanodine receptor Ca^{2+}-release channel.

Before the analysis of Ca^{2+}-regulatory elements, we wanted to establish the general fiber-type distribution in the specimens investigated. The isoform expression patterns of the SR Ca^{2+}-ATPase and the MHC both demonstrated a heterogeneous fiber population in the investigated muscle specimens. The SR from MHS individuals is believed to recycle Ca^{2+} at a higher rate, as efflux from MH RyR1 Ca^{2+}-release channels is continuously elevated compared with normal muscle, even during relaxation (42). This, however, does not appear to result in increased levels of SERCA expression. The slow and fast MHC isoforms are good indicators of general fiber-type distribution in total human skeletal muscle extracts (8), but in SR samples the interpretation of their expression levels is complicated because of the fact that both proteins probably represent a heterogeneous mixture of myosin species within vesicular membrane preparations. We did not observe dramatic differences in the distribution of slow and fast MHC in normal control and MHS specimens, which indicates that the biopsied samples do not exhibit major differences in their fiber-type composition. Thus our immunoblot survey was not influenced by differences in isoform expression patterns. However, MHC species found in muscle microsomes originally derive from both the homogenized contractile apparatus and a molecular subpopulation of “easy-releasable” myosins. The secondary attachment and/or entrapment of these proteins in microsomal vesicles during tissue homogenization and subcellular fractionation cannot be sufficiently controlled. Therefore, one cannot conclusively determine the exact fiber-type composition of the biopsy sample from the relative content of the fast and slow MHC isoforms present in the microsomal vesicle preparation. However, one can exclude major differences between two analyzed samples.

An abundant cytosolic enzyme that is potentially associated with the triadic Ca^{2+}-release channel complex is represented by glyceraldehyde 3-phosphate dehydrogenase (6, 34). We could recently confirm the electrostimulation-induced decrease in the expression of this marker of anaerobic-glycolytic energy metabolism during fiber-type shifting (44) by immunoblotting (41). Thus changes in the level of key enzymes involved in energy metabolism can be used to detect changes in the fiber-type composition of muscle biopsies. Here we could show that the expression of glyceraldehyde 3-phosphate dehydrogenase is not affected in MH, indicating that the fulminant hypermetabolic crisis observed in MH is not attributable to an increase in this metabolic enzyme culminating in an enhanced capacity of diseased fibers for glycolysis. It also shows that its expression is not influenced by potential changes in its association to a mutated triad complex in MHS muscle.

Specific mutations in the RyR1 isoform of the SR Ca^{2+}-release channel alone are sufficient to confer the autosomal dominant MHS trait in a large percentage of MH families (33). Because the ryanodine receptor does not exist in isolation in the junctional triad membrane (38) but is closely associated with other SR elements (20, 30), a mutated RyR1 receptor could potentially trigger changes in the relative abundance of other ion-regulatory proteins. However, our immunoblotting survey did not detect a difference in the abundance or electrophoretic mobility of the RyR and its endogenous regulators junctin, the FK506 binding protein of 12 kDa, and calmodulin. The fact that the relative density of CSQ, CLPs, sarcalumenin, and the 53-kDa SR glycoprotein are unaffected in MHS samples suggests that the decreased loading ability of MHS SR vesicles (42) is not due to the depressed expression of key Ca^{2+}-reservoir elements, but more likely a feature of altered quaternary receptor structure and/or modified functional dynamics of key Ca^{2+}-binding proteins. Mutations in the dihydropyridine receptor are also implicated in the molecular pathogenesis of MH (35), and because this transverse-tubular protein directly interacts with the ryanodine receptor during excitation-contraction coupling (28), it is interesting that the expression of both α-subunits of the voltage-sensing dihydropyridine receptor are comparable in MHS and MHN. This confirms the supposition that the MHS phenotype is attributable to aberrant Ca^{2+} channel function although not necessarily due to abnormal expression levels of triadic Ca^{2+} regulatory proteins. Cytoplasmic annexin VI is an accessory protein of the supramolecular triad complex involved in excitation-contraction coupling (24). Our immunoblotting suggests that susceptibility to MH does not change its expression profile.

Hypersensitive gating of the major SR Ca^{2+} release channel appears to be the underlying defect in cases linked to the RyR1 MHS locus (9). The volatile anesthetics are noteworthy among the drugs that may increase RyR1 activity and perturb Ca^{2+} regulation, especially in MHS individuals. Although normal skeletal muscle may tolerate a modest perturbation of Ca^{2+} homeostasis, such as increased Ca^{2+} leak from the SR, without phenotypic evidence of a problem, myopathic MH muscle that exhibits a leaky SR membrane because of mutated RyR1 Ca^{2+} release channels may be compensated normally until perturbed by an agent that further activates Ca^{2+} release, thus further straining homeostatic mechanisms (43). Because halothane has a modulatory effect on SR Ca^{2+} release at clinical concentrations and has previously been shown to induce oligomerization of several key SR components (7, 17, 18), the effects of anesthetic complexation were investigated in MHN and MHS SR vesicles. Our study clearly shows a reduced electrophoretic mobility of halothane-treated RyR1. Possibly, stabilization of the open channel conformation by halothane or aggregation of the RyR1 channel complexes themselves may account for this oligomerization pattern.

In analogy to findings reported on the cardiac isoform of the SR Ca^{2+} ATPase (25), halothane-induced aggregation of SERCA molecules appears to inhibit enzyme functioning, particularly in MHS SR vesicles at clinically relevant drug concentrations. Previous studies suggest that the biomechanical mechanism of halothane inhibition of Ca^{2+} pump function involves stabilization of an intermediate enzyme conformation (26, 32). Halothane-mediated clustering might introduce conformational changes that inhibit proper subunit interactions, thereby decreasing positive cooperativity within the physiological Ca^{2+} pump unit of the SR membrane (7, 26).

In conclusion, the findings of this study support two ideas: *1*) volatile anesthetics appear to directly influence sensitive protein-protein interactions within integral membrane protein complexes and *2*) the development of an anesthetic-mediated crisis of MH may involve RyR1 protein complex formation. Aberrant RyR1 channel function, resulting from mutations within the amino acid sequence in MHS individuals, may be exacerbated by such an anesthetic-mediated channel conformational change, inducing prolonged opening of the Ca^{2+} channel with reduced sensitivity to inactivating agents and resultant elevation of myoplasmic Ca^{2+} levels. In addition, the results presented here also suggest a possible auxiliary role of depressed SERCA activity in the molecular pathogenesis of MH. Reduced resequestration of Ca^{2+} ions back into the SR lumen may contribute to sustained elevation of cytoplasmic Ca^{2+} ions and thus exacerbate metabolic activation and sustained muscle contraction during an MH episode.

The sequence of pathophysiological events leading to halothane-induced MH may be divided into the following steps. In a normal individual, the Ca^{2+}-release channel complex is regulated by direct coupling to the transverse-tubular dihydropyridine receptor, local Ca^{2+} levels, ATP, and various endogenous protein factors such as calmodulin, triadin, and CSQ. Receptor stimulation triggers only transient channel opening, and drug-induced disturbances of Ca^{2+} homeostasis are quickly rectified by the efficient Ca^{2+} recycling via the action of pumps, transporters, and binding proteins. In MH, the mutated Ca^{2+} channel exhibits an increased sensitivity to activating agents, and clinical concentrations of volatile anesthetics trigger a prolonged channel opening. The enhanced efflux of Ca^{2+} ions from the SR, together with a decreased Ca^{2+}-uptake rate, results in a prolonged elevation of cytosolic Ca^{2+} levels. This in turn triggers sustained muscle contraction, metabolic activation, and membrane damage, resulting in the main symptoms of malignant hyperthermia, i.e., muscle rigidity, fiber damage, and heat production.

Although the pharmacological actions of inhalational anesthetics have not yet been classified in the general scheme of receptor-mediated drug action, this study agrees with the hypothesis that distinct binding sites exist in target proteins for halothane. In contrast to the conventional view that general anesthetics trigger nonspecific perturbation of biological membranes, this study suggests that substances that may trigger surgical anesthesia probably interact through specific hydrophobic binding sites within receptor complexes. The protein gel-shift experiments presented here demonstrate that halothane can directly interfere with the oligomeric status of the Ca^{2+}-release channel complex. This clearly supports the protein theory of anesthetic action and sets the scene for the elucidation of the binding domains involved in the action of volatile anesthetics.

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