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Distinct Immunopeptide Maps of the Sarcoplasmic Reticulum Ca^{2+} Release Channel in Malignant Hyperthermia*

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Sarcoplasmic reticulum isolated from malignant hyperthermia-susceptible (MHS) muscle exhibits abnormalities in the regulation of calcium release. To identify the molecular basis of this abnormality, the Ca^{2+} release channel from both normal and MHS sarcoplasmic reticulum was examined using proteolytic digestion followed by immunoblot staining with a polyclonal antibody against the rabbit Ca^{2+} release channel protein. Under appropriate conditions, trypsin digestion of isolated sarcoplasmic reticulum vesicles from the two types of pigs revealed a distinct difference in the immunostaining pattern of the Ca^{2+} release channel-derived peptides. An approximate 86-kDa peptide was the predominant fragment in normal sarcoplasmic reticulum while an approximate 99-kDa peptide fragment was the major peptide detected in MHS sarcoplasmic reticulum. Digestion of sarcoplasmic reticulum vesicles isolated from four normal and four MHS pigs showed that the differences were highly reproducible. Trypsin digestion of sarcoplasmic reticulum isolated from heterozygous pigs, which contain one normal and one MHS allele, showed an antibody staining pattern that was intermediate between MHS and normal sarcoplasmic reticulum. These results can be explained by a primary amino acid sequence difference between the normal and MHS Ca^{2+} release channels and support the hypothesis that a mutation in the gene coding for the sarcoplasmic reticulum Ca^{2+} release channel is responsible for malignant hyperthermia.

Malignant hyperthermia (MH)[1] is an inherited skeletal muscle disorder of humans and pigs in which halogenated anesthetics, by elevating intracellular Ca^{2+}, induce an accelerated muscle metabolism and muscle contracture (1, 2). An MH episode can result in death unless promptly recognized and treated with the skeletal muscle relaxant dantrolene (3). Although the exact cause of the explosive rise in sarcoplasmic Ca^{2+} is not completely understood, several laboratories have found that Ca^{2+} release from MHS sarcoplasmic reticulum differs from that of normal sarcoplasmic reticulum in both isolated vesicles (4–8) as well as intact or skinned fibers (9–13).

The skeletal muscle receptor for the plant alkaloid ryanodine (14) has been purified and shown to be identical to the Ca^{2+} release channel of the sarcoplasmic reticulum (15–17). This protein is thought to compose the "foot" structures which are present at the junction between the sarcoplasmic reticulum and the transverse tubule membranes (17, 18). The foot structures may interact with the recently identified transverse tubule tetrad which has been proposed to be the dihydropyridine receptor (18). Recent results by Mickelson *et al.* (19) provide evidence that the sarcoplasmic reticulum Ca^{2+} release channel/ryanodine receptor itself may be altered in MH. These studies showed that the MHS Ca^{2+} release channel has an altered Ca^{2+} dependence of ryanodine binding and a higher affinity for ryanodine than does the normal Ca^{2+} release channel protein.

The primary amino acid sequence for the skeletal muscle Ca^{2+} release channel has been deduced from the cDNA (20). It is predicted to contain four transmembrane domains near the carboxyl terminus, with the majority of the protein (M_r = 565,223) protruding into the cytoplasm to form the "foot" (20). Consistent with the above model, several groups have shown that the Ca^{2+} release channel is highly susceptible to proteolytic degradation (21–24), resulting in multiple peptide fragments which can be identified by immunoblot analysis (22). Based on results using electron microscopy (23) and sucrose gradient sedimentation (22) it appears that despite extensive digestion by trypsin, the channel/foot protein remains intact through noncovalent interactions.

In this study we have tested the proposal that differences between the structure of the MHS and normal sarcoplasmic reticulum Ca^{2+} release channels may be identified by immunoblot analysis as an altered proteolytic digestion pattern. This procedure allows for the unique identification of subtle differences between normal and mutated proteins, and we propose this technique be called immunopeptide mapping. We report that when sarcoplasmic reticulum vesicles were digested with trypsin, clear and reproducible differences were seen between the immunostaining pattern of the MHS and normal Ca^{2+} release channel-derived peptides. These data support the proposal that MH results from a mutation in the gene coding for the sarcoplasmic reticulum Ca^{2+} release channel.

EXPERIMENTAL PROCEDURES

Isolation of Normal, Heterozygote, and MHS Sarcoplasmic Reticulum—MHS Pietrain pigs (homozygous for the halothane sensitivity (MHS) gene), heterozygote pigs (one normal and one MHS allele), and normal Yorkshire (homozygous for the normal allele) pigs (60–100 pounds) from the University of Minnesota Experimental Farm were evaluated for malignant hyperthermia susceptibility by a halothane challenge test 3–4 weeks before use (25). Heavy sarcoplasmic reticulum was isolated as described previously (4, 19). Protein was

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[1] The abbreviations used are: MH, malignant hyperthermia; MHS, malignant hyperthermia susceptible; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

determined by the method of Lowry *et al.* (26) as modified by Peterson (27).

Protease Digestion of Isolated Sarcoplasmic Reticulum—Isolated heavy sarcoplasmic reticulum was diluted with Buffer A (0.3 M sucrose, 20 mM Tris-HCl, pH 7.4) and centrifuged for 30 min at 100,000 × *g* to remove protease inhibitors. The sarcoplasmic reticulum pellet was resuspended in Buffer A, frozen in liquid nitrogen, and stored at −135 °C until use. Sarcoplasmic reticulum (1 mg/ml) was digested with protease at either 30 or 37 °C for various times in a buffer containing 1.0 M sucrose and 20 mM Tris-HCl, pH 7.0. Digestion was inhibited by the addition of 1 mM phenylmethylsulfonyl fluoride followed by an equal volume of Laemmli sample buffer (28) containing 6% SDS and 2% 2-mercaptoethanol.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis—Skeletal muscle membranes were analyzed by SDS-PAGE (3–12 or 5–16% gradient gels) using the buffer system of Laemmli (28) and transferred to nitrocellulose membranes according to Towbin *et al.* (29). The nitrocellulose blots were blocked for 1 h with phosphate-buffered saline (154 mM NaCl, 50 mM NaH_2PO_4, pH 7.4 with NaOH) containing 5% nonfat dry milk and subsequently incubated with primary antibody in Tris-buffered saline (TBS) (200 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 3% bovine serum albumin overnight at 4 °C. Immunoblots were then washed three times with TBS, incubated for 1 h with secondary anti-IgG antibodies covalently linked to horseradish peroxidase in TBS containing 3% bovine serum albumin, washed with TBS, and finally developed using 4-chloro-1-naphthol as the substrate.

Polyclonal antibodies against the sarcoplasmic reticulum Ca^{2+} release channel were prepared by injection of a sheep with 0.5 mg of purified Ca^{2+} release channel (15) in 5 ml of Freund's complete adjuvant. The sheep was boosted 2, 4, and 6 weeks later with another 0.5 mg of purified Ca^{2+} release channel protein in 5 ml of Freund's incomplete adjuvant. Between 3 and 10 weeks later blood was collected, incubated first at 37 °C for 1 h and then at 4 °C overnight. The serum was collected by centrifugation, then tested for specificity and titered using immunoblots containing both purified Ca^{2+} release channel and crude membranes.

Materials—Electrophoresis reagents were obtained from Bio-Rad and molecular weight standards from Bethesda Research Laboratories. Trypsin (T-8642) (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) and phenylmethylsulfonyl fluoride were purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies were obtained from Boehringer Mannheim. All other chemicals were of reagent grade quality.

RESULTS AND DISCUSSION

To explore potential differences in the structure of the MHS and normal Ca^{2+} release channel, heavy sarcoplasmic reticulum vesicles were treated with various proteases and examined for differences in the pattern of proteolytic digestion. When samples were analyzed by Coomassie Blue or silver staining of SDS-PAGE gels, no differences were seen between the MHS and normal samples (data not shown). Therefore, the samples were analyzed by SDS-PAGE, followed by immunoblotting with polyclonal antibodies against the Ca^{2+} release channel protein. This methodology is more sensitive and specific than analysis by gel staining and may therefore detect subtle differences in the protease digestion patterns not seen by gel staining alone. Additionally, this method allows the digestion of the samples under nondenaturing conditions, where the effects of ligands or phosphorylation on the digestion pattern could be studied. Four different concentrations of either trypsin, α-chymotrypsin, or *Staphylococcus aureus* protease V8 were used. No reproducible differences in MHS and normal immunostaining of the Ca^{2+} release channel were identified when either α-chymotrypsin or *S. aureus* protease was used (data not shown). However, Fig. 1 shows that when the samples were digested with trypsin, differences were detected in the staining pattern of the immunoblot. At a protease:protein ratio of 1:160 (6.25 ng/μg) the MHS sample contained an approximate 99-kDa band which was only a minor band in the normal sample, while the normal sample contained an 86-kDa band which was not

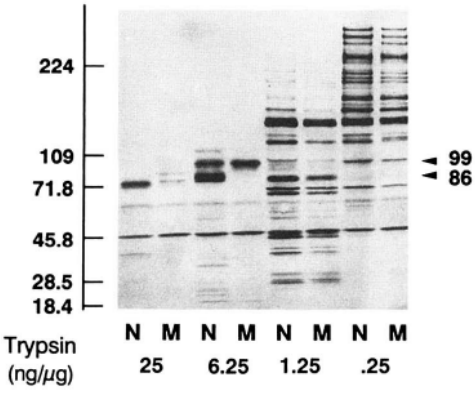

FIG. 1. **Immunoblot of MHS and normal sarcoplasmic reticulum digested with various concentrations of trypsin.** Each *lane* contains 50 μg of either normal (*N*) or MHS (*M*) sarcoplasmic reticulum treated with trypsin for 5 min at 37 °C and analyzed on 3–12% SDS-polyacrylamide gels. The immunoblot was stained with sheep polyclonal anti-rabbit Ca^{2+} release channel antiserum followed by incubation with rabbit anti-sheep IgG conjugated to horseradish peroxidase. The amount of protease (ng) per μg of sarcoplasmic reticulum protein is indicated at the *bottom*. The *arrowheads* on the *right* indicate the 86-kDa fragment which is detected in the normal sarcoplasmic reticulum but not in the MHS and the 99-kDa fragment which is prominent in the MHS sarcoplasmic reticulum. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the *left*. (Prestained molecular weight standards were from Bethesda Research Laboratories and the apparent molecular weights were as follows: myosin, 224,330; phosphorylase *b*, 109,100; bovine serum albumin, 71,830; ovalbumin, 45,830; carbonic anhydrase, 28,530; and β-lactoglobulin, 18,370.)

detected in the MHS sample. These differences were not seen at lower trypsin concentrations (1.25 and 0.25 ng/μg in Fig. 1), indicating that the differences in digestion of the MHS and normal Ca^{2+} release channel may only be detectable over a limited range of trypsin concentrations.

Control experiments using antibodies against the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-ATPase, the junctional transverse tubular dihydropyridine receptor (30), and a junctional sarcoplasmic reticulum 94-kDa protein (31) revealed no differences in the tryptic digestion pattern of MHS and normal sarcoplasmic reticulum (data not shown). Immunopeptide maps of normal and MHS sarcoplasmic reticulum revealed differences only in the Ca^{2+} release channel. One interpretation of these results is that the Ca^{2+} release channel of MHS sarcoplasmic reticulum contains one or more trypsin-susceptible peptide bonds which are either absent or less accessible in the Ca^{2+} release channel of normal sarcoplasmic reticulum.

To demonstrate consistent differences in the trypsin sensitivity of the MHS and normal Ca^{2+} release channel, four different preparations each of MHS and normal sarcoplasmic reticulum were treated with trypsin at a ratio of 160:1 for 5 min at 37 °C (Fig. 2). Again, it can be seen that more of the 99-kDa fragment is present in each of the MHS samples while the 86-kDa fragment is seen in each of the normal samples. This experiment shows that the differences in digestion patterns between MHS and normal samples cannot be accounted for by pig-to-pig or preparation-to-preparation variability.

To explore further the nature of the differences in the pattern of trypsin digestion, MHS and normal samples were treated for various times at a trypsin:protein ratio of 1:160 at 30 °C (Fig. 3). Clear differences were again seen in the immunostaining patterns of the MHS and normal Ca^{2+} release channel peptides. However, this experiment shows that both MHS and normal samples contain a 99-kDa fragment at short incubation times (5–15 min). Fig. 3 also shows that while the

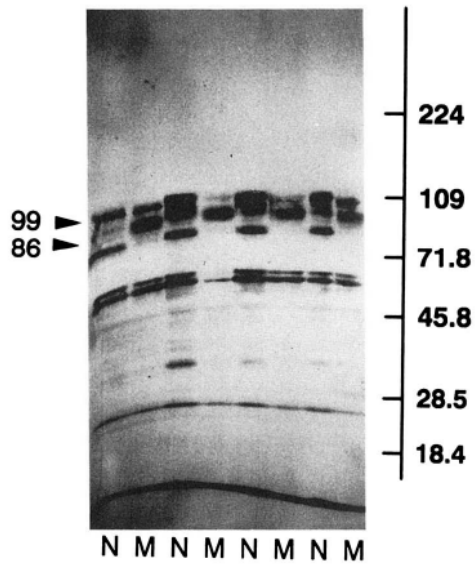

FIG. 2. **Immunoblots of different preparations of MHS and normal sarcoplasmic reticulum digested with trypsin.** Samples were treated with trypsin for 5 min at 37 °C as described under "Experimental Procedures" and analyzed using a 5–16% SDS-polyacrylamide gel. The ratio of sarcoplasmic reticulum to trypsin was 160:1. The immunoblot was stained with polyclonal sheep anti-rabbit Ca^{2+} release channel antibodies followed by incubation with rabbit anti-sheep IgG conjugated to horseradish peroxidase. Each *lane* contains 100 μg of either normal (*N*) or MHS (*M*) sarcoplasmic reticulum. The *arrowheads* on the *left* show the 86-kDa fragment which is detected in the normal sarcoplasmic reticulum but not in the MHS and the 99-kDa fragment which is prominent in the MHS sarcoplasmic reticulum. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the *right*. (Prestained molecular weight standards are as in Fig. 1.)

99-kDa normal fragment was almost completely absent by 30 min, the 99-kDa MHS fragment is only partially lost after 60 min of digestion; this is associated with the simultaneous appearance of an approximate 86-kDa fragment. Whether the 86-kDa immunostained band that is visible in the 30- and 60-min MHS samples is the same as the 86-kDa band seen after 5 min of trypsin incubation with the normal sample remains to be determined. However, it is clear that this 86-kDa Ca^{2+} release channel fragment is produced at very different times of trypsin incubation with MHS and normal sarcoplasmic reticulum.

In addition to the Yorkshire and the Pietrain pigs, which are homozygous normal and MHS, respectively, heterozygotes have been developed by cross-breeding of the two genotypes and backcrossing to Pietrains (32). The heterozygote pigs were found to be phenotypically non-MHS when challenged with halothane, consistent with the MHS gene being inherited in an autosomal recessive fashion (32). An advantage of these animals is that except for the halothane sensitivity gene, most of the genes will be provided from the Pietrain breed. However, sarcoplasmic reticulum isolated from heterozygote displayed intermediate properties of Ca^{2+} release and ryanodine binding activities when compared with the homozygous normal and homozygous MHS pigs (33). Fig. 4 shows the trypsin digestion pattern of the sarcoplasmic reticulum Ca^{2+} release channel from one normal, three heterozygous, and one MHS pig. Under conditions where the predominant MHS and normal fragments are 99 and 86 kDa, respectively, the heterozygote samples display both bands. Thus, the sarcoplasmic reticulum Ca^{2+} release channel of heterozygous pigs demonstrates an intermediate pattern of trypsin digestion. This would be expected if the halothane sensitivity gene codes for

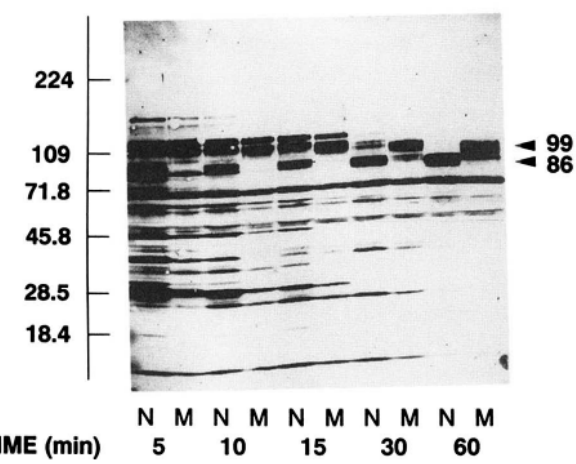

FIG. 3. **Time course of trypsin digestion of MHS and normal sarcoplasmic reticulum.** Samples were treated with trypsin as described under "Experimental Procedures" and analyzed using a 5–16% SDS-polyacrylamide gel. The immunoblot was stained with sheep polyclonal anti-rabbit Ca^{2+} release channel antibodies followed by incubation with rabbit anti-sheep IgG conjugated to horseradish peroxidase. Each lane contains 100 μg of either normal (*N*) or MHS (*M*) sarcoplasmic reticulum treated with 6.35 ng/μg sarcoplasmic reticulum protein or a ratio of 160:1 (sarcoplasmic reticulum:trypsin) at 30 °C. The *arrowheads* on the *right* show the 86-kDa fragment which is detected in the normal sarcoplasmic reticulum but not in the MHS sarcoplasmic reticulum and the 99-kDa fragment which is prominent in the MHS sarcoplasmic reticulum. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the *left*. (Prestained molecular weight standards are as in Fig. 1.)

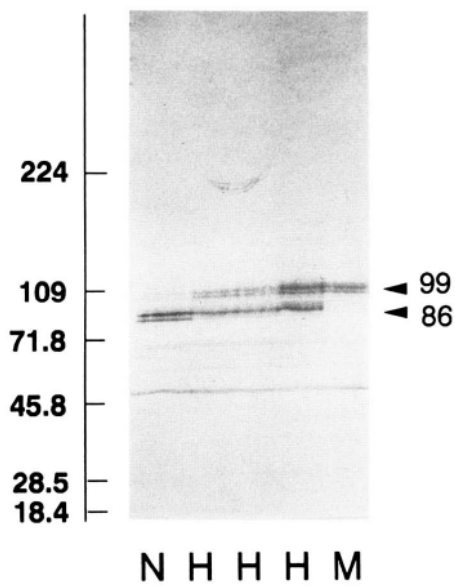

FIG. 4. **Immunoblots of MHS (*M*), heterozygote (*H*), and normal (*N*) sarcoplasmic reticulum digested with trypsin at a ratio of 160:1 sarcoplasmic reticulum:trypsin.** Samples were treated with trypsin as described under "Experimental Procedures" for 5 min at 37 °C, after which 75 μg/lane were analyzed on 3–12% SDS-PAGE. The nitrocellulose transfer was stained with polyclonal sheep anti-rabbit Ca^{2+} release channel antibody followed by incubation with rabbit anti-sheep IgG conjugated to horseradish peroxidase. The *arrowheads* on the *right* show the 86-kDa fragment which is detected in the normal sarcoplasmic reticulum but not in the MHS and the 99-kDa fragment which is prominent in the MHS sarcoplasmic reticulum. Molecular weight standards ($M_r \times 10^{-3}$) are indicated on the *left*. (Prestained molecular weight standards are as in Fig. 1.)

the sarcoplasmic reticulum Ca^{2+} release channel, *i.e.* pigs heterozygous for the halothane sensitivity gene contain one copy each of the normal and altered alleles of the sarcoplasmic reticulum Ca^{2+} release channel gene. The results with the heterozygotes also suggest that the differences in immunopeptide maps of the MHS and normal sarcoplasmic reticulum Ca^{2+} release channel are not due to other genes which may differ between the two breeds of pigs.

The most likely explanation for our observations is that the halothane sensitivity/MHS gene is identical to the Ca^{2+} release channel gene, with a mutation in this gene directly responsible for the different peptide maps of the MHS and normal Ca^{2+} release channel proteins. The MH mutation may result in the direct loss/gain of trypsin sites (arginine or lysine). One recent example of this type of mutation is illustrated by the recent cloning of the gene responsible for cystic fibrosis (34); 70% of the cystic fibrosis cases were found to be the result of the deletion of a single amino acid (35). Clearly, any mutation in the Ca^{2+} release channel (deletion, insertion, or substitution) may result in an altered secondary, tertiary, or quaternary structure of the protein which could alter the immunopeptide map with trypsin. A mutation which results in a change in structure of the MHS channel may also explain the greater open channel probability (36), the altered [^{3}H] ryanodine binding (19), and the increased Ca^{2+}-induced Ca^{2+} release seen in MHS sarcoplasmic reticulum (4–8).

An alternative explanation for these results could be that the MHS mutation only indirectly affects the Ca^{2+} release channel, *i.e.* the halothane sensitivity gene may encode for another protein involved in excitation-contraction coupling, which then interacts differently with the MHS and normal Ca^{2+} release channel proteins. Although the purified Ca^{2+} release channel is thought to be a homotetramer (17, 18), it is still possible that other proteins interact with the channel *in situ* but are lost when the channel is solubilized and purified. However, we have found no differences in immunopeptide maps for two other potential components of excitation-contraction coupling, the junctional transverse tubule dihydropyridine receptor or junctional sarcoplasmic reticulum 94-kDa protein. Regardless of the explanation for the differences between MHS and normal, it is clear that immunopeptide mapping provides another way to distinguish between MHS and normal pigs.

In this report we have shown specific and distinct differences in the immunopeptide maps of the Ca^{2+} release channel between MHS and normal pigs. These different immunopeptide maps are most likely the result of specific differences in the amino acid sequences of the two proteins. Furthermore, differences in the amino acid sequence of the Ca^{2+} release channel could cause structural differences in the two proteins which may account for the abnormal regulation of sarcoplasmic reticulum Ca^{2+} release seen in MH. Since the COOH-terminal one-fourth of the protein is proposed to contain the regulatory and transmembrane domains (20), this region is an excellent candidate for the site of the MHS mutation. In conclusion, although alternative interpretations exist, we propose the most likely explanation of our results is that the gene encoding the Ca^{2+} release channel is the candidate gene responsible for MH.

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