Inhibition of Ubiquitin Proteasome System Rescues the Defective Sarco(endo)plasmic Reticulum Ca\textsuperscript{2+}\textsubscript{-ATPase} (SERCA1) Protein Causing Chianina Cattle Pseudomyotonia**

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A missense mutation in ATP2A1 gene, encoding sarco(endo)plasmic reticulum Ca\textsuperscript{2+}\textsubscript{-ATPase} (SERCA1) protein, causes Chianina cattle congenital pseudomyotonia, an exercise-induced impairment of muscle relaxation. Skeletal muscles of affected cattle are characterized by a selective reduction of SERCA1 in sarcoplasmic reticulum membranes. In this study, we provide evidence that the ubiquitin proteasome system is involved in the reduced density of mutated SERCA1. The treatment with MG132, an inhibitor of ubiquitin proteasome system, rescues the expression level and membrane localization of the SERCA1 mutant in a heterologous cellular model. Cells co-transfected with the Ca\textsuperscript{2+}\textsubscript{-sensitive probe aequorin show that the rescued SERCA1 mutant exhibits the same ability of wild type to maintain Ca\textsuperscript{2+} homeostasis within cells. These data have been confirmed by those obtained ex vivo on adult skeletal muscle fibers from a biopsy from a pseudomyotonia-affected subject. Our data show that the mutation generates a protein most likely corrupted in proper folding but not in catalytic activity. Rescue of mutated SERCA1 to sarcoplasmic reticulum membrane can re-establish resting cytosolic Ca\textsuperscript{2+} concentration and prevent the appearance of pathological signs of cattle pseudomyotonia.

**Significance:** The data suggest a therapeutic approach against Brody disease.

Cattle “congenital pseudomyotonia” (PMT)\textsuperscript{3} is a muscular disorder described for the first time in the Italian Chianina breed by Testoni et al. (1), characterized by an impairment of muscle relaxation induced by exercise. When animals are stimulated to perform intense muscular activities, muscles become stiff and freeze up temporarily, inducing a rigid gait. If the exercise is prolonged, the sustained contraction immobilizes the affected animal, which eventually falls down. After a few seconds at rest, the stiffness disappears, and the animal regains the ability to stand up and move. By DNA sequencing of affected Chianina cattle, we provided evidence of a missense mutation in exon 6 of ATP2A1 gene, encoding sarco(endo)plasmic reticulum Ca\textsuperscript{2+}\textsubscript{-ATPase} (SERCA) isoform 1 (2). SERCA, the main protein component of the non-junctional sarcoplasmic reticulum (SR) (3), is a key participant in the Ca\textsuperscript{2+} homeostasis in skeletal muscle fibers, being responsible for pumping Ca\textsuperscript{2+} from cytosol back into SR lumen, thus initiating relaxation. In skeletal muscle fibers, Ca\textsuperscript{2+}-activating muscle contraction is released from the SR lumen into the cytosol via Ca\textsuperscript{2+} release channel localized at the terminal cisternae of SR. At the end of the contraction cycle, SERCA allows relaxation by removing Ca\textsuperscript{2+} from the cytosol to restore resting Ca\textsuperscript{2+} concentration. Three SERCA isoforms, products of different genes, are expressed in striated muscles in a tissue- and stage of development-specific fashion. SERCA1 isoform is expressed in fast-twitch (type 2) skeletal muscle of mammals (4).

The mutation underlying Chianina cattle PMT replaces an Arg at position 164 by His (R164H), in a highly conserved region of the Actuator (A) domain of SERCA1 protein (5). This
Inhibition of Proteasome Rescues Defective SERCA1

mutation does not affect the expression of ATP2A1 gene as SERCA1 mRNA levels found in affected animals are comparable with mRNA expression in normal samples (6). However, Chianina pathological muscles are characterized by a striking, selective reduction in the expression level of SERCA1 protein (6). Although present at low levels, the R164H SERCA1 variant maintained the basic intrinsic properties of WT SERCA1, notably the Ca²⁺-dependent ATPase activity. Therefore, we concluded that the decrease in SR Ca²⁺-ATPase activity found in affected animals was mainly due to reduction of SR SERCA1 protein content (6). The consequent reduction in pumping efficiency of SR is likely responsible for muscle stiffness as the abnormally low rate of Ca²⁺ removal from the cytosol supports an elevated cytoplasmic Ca²⁺ concentration, thereby triggering contractures.

More recently, cattle PMT associated with ATP2A1 gene mutations different from that of Chianina breed has been described in Romagnola breed (7), as a single case in a Dutch improved Red and White cross-breed calf (8), and in the Belgian Blue breed. (In these cases, the disease was called “congenital muscular dystonia” (9).

The relevance of these animal models resides in the similarity of the clinical phenotype to that of human Brody disease (10), a rare inherited disorder of skeletal muscle due to SR Ca²⁺-ATPase deficiency, resulting from a defect of ATP2A1 gene (11). Clinical key features are exercise-induced muscle stiffness and delayed muscle relaxation after repetitive contraction. The muscular stiffness observed in Brody disease patients is currently thought to be due to a deficiency of SERCA1 protein at SR membranes, which causes a reduced uptake of Ca²⁺ into the lumen of SR after sustained exercise (12). Like cattle congenital PMT, Brody disease is genetically heterogeneous (13). Therefore, based on clinical presentation and genetic and biochemical findings, it is possible to deem Chianina cattle congenital PMT as a true counterpart of human Brody disease. Thus, Chianina PMT is a very useful, although unconventional, model for the study of myopathy in human Brody disease and for the development of innovative therapeutic approaches. The observation that in cattle SERCA1 mRNA levels in diseased muscles are normal while protein levels are markedly reduced suggested to us that the R164H mutation could cause SERCA1 misfolding and accelerated removal by either the ubiquitin-proteasome system (UPS) or the autophagic-lysosomal pathway.

In this study, we have investigated the possible involvement of the UPS in the reduced levels of mutated SERCA1 in SR from Chianina PMT muscles. Our results provide strong support to this interpretation.

EXPERIMENTAL PROCEDURES

SERCA1 Construct and Site-directed Mutagenesis—The original full-length rabbit neonatal SERCA1 cDNA clone was a kind gift of Prof. D. MacLennan (14). To obtain the adult full-length SERCA1 isoform, in which the C-terminal extension of the neonatal form has been removed, a PCR was carried out with the following primers: forward, 5’-CAAGAAGCTTGGCAGAATG- GAG, and reverse, 5’-CTTTGAAATCTTAACCTCCAGGTAGTTC. The forward primer contains a HindIII restriction site (in bold in the primer sequence) upstream to the ATG codon to facilitate cloning. The reverse primer permits us to change the triplet coding for Glu¹⁹³ to a Gln (in italics in the primer sequence); the following triplet is in a STOP codon (underlined), and it introduces an EcoRI restriction site (in bold) to facilitate cloning. After restriction digestion, the amplified fragment was cloned in pcDNA3.1 expression vector. The R164H SERCA1 mutant was obtained by site-directed mutagenesis according to the manufacturer’s instructions (Stratagene) (15). The constructs were verified by sequencing.

Cell Culture, Transfection, and Treatment with Proteasome Inhibitors—HEK293 cells were counted using the automated cell counter Scepter (Merck Millipore) and then were seeded and grown in DMEM high glucose medium supplemented with 10% FCS. Cells were transfected using the TransIT-T293 transfection reagent (Mirus Bio), according to manufacturer’s instructions. Sixteen hours after transfection, cells were incubated for 8 h with the proteasome inhibitors MG132 (Sigma) (10 μM final concentration), lactacystin (Sigma) (20 μM final concentration), or bortezomib (Sigma) (0.5 μM final concentration), and then dissolved in DMSO.

SERCA1 Half-life Determination—HEK293 cells were transfected with WT and R164H SERCA1 mutant cDNAs. Twelve hours after transfection, cells were incubated for 4 and 8 h with cycloheximide (Sigma) (40 μg/ml, final concentration).

Muscle Biopsies—Biopsy of semimembranosus muscle, a representative fast-twitch skeletal muscle (6), was collected from PMT-affected Chianina animal under local anesthesia during the course of routine clinical investigation, in compliance with Italian law. Biopsy was divided in small bundles of fibers, placed in dishes containing Ham’s F14 supplemented with 15% FCS, 50 μg/liter FGF, 10 mg/liter insulin, and 10 μg/liter EGF, and incubated for 24 h with the proteasome inhibitor MG132 (20 μM) (16). Control muscle samples were collected from healthy Chianina cattle euthanized at a slaughterhouse.

Transmission Electron Microscopy Analysis—Immediately after collection, muscle samples were fixed with 4% paraformaldehyde and 2% glutaraldehyde in 0.12 M phosphate buffer and post-fixed with 1% OsO₄ in cacodylate buffer, dehydrated in ethanol, and embedded in epoxy resin. Ultrathin sections, obtained with an Ultracut E Reichert-Jung ultramicrotome, were doubly stained with uranyl acetate and lead citrate and examined by a CM 10 Philips transmission electron microscope (FEI, Eindhoven, the Netherlands).

Immunocytochemical Analysis—HEK293 cells grown on 13-mm glass coverslips, were washed with PBS, pH 7.4, and fixed for 10 min in 4% paraformaldehyde and then washed with PBS. The cells were permeabilized in 0.5% Triton X-100 in PBS and incubated with the following primary antibodies: mouse monoclonal antibody to SERCA1 (Biomol) (recognizing an epitope between amino acid residues 506 and C terminus of rabbit fast-twitch SR Ca²⁺-ATPase) (dilution 1:500); and rabbit polyclonal antibody to calreticulin (Affinity Bioreagents) (dilution 1:100). Cells were then incubated with the appropriate secondary antibody conjugated with TRITC or FITC (Dako). Confocal microscopy was performed using a Leica TCS-SP2 confocal laser scanning microscope.

Preparative Procedures—At the end of the incubation time, transfected HEK293 cells treated with MG132, lactacystin,
Inhibition of Proteasome Rescues Defective SERCA1

bortezomib, and vehicle, or with cycloheximide, were washed twice with PBS. Total cell lysate was obtained by solubilization in 5% sodium deoxycholate containing protease inhibitors (Roche Applied Science). The isolation of microsomal fraction from HEK293 cells was carried out according to Ref. 14, from five 10-cm Petri dishes for each sample. The crude membrane fraction, enriched in content of SR membranes, was isolated by differential centrifugations from muscle biopsy, as described previously (6). Protein concentration was determined by the method of Lowry et al. (17).

**Gel Electrophoresis Immunoblotting and Biochemical Assay**—SDS-PAGE and immunoblotting were carried out as described (6). The blots were probed with mouse monoclonal antibodies to: SERCA1 (1:5000); β-actin (1:30000) (Sigma); and mono- and polyubiquitinated conjugates (1:1000) (Enzo Life Science). Densitometric scanning of protein bands was carried out using the ImageQuant 350 (GE Healthcare). ATPase activity of the microsomal fractions (20 µg/ml) was measured by spectrophotometric determination of NADH oxidation coupled to an ATP-regenerating system, as described previously (6).

**Immunoprecipitation**—HEK293 cells were washed with PBS and subsequently lysed in radioimmunoprecipitation assay solubilization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA) supplemented with 2 mM N-ethyl-maleimide (Sigma) and protease inhibitors. A soluble supernatant and a pellet were collected after spinning for 30 min at 10000 × g, at 4 °C. The lysate was incubated overnight while tumbling at 4 °C with the specific antibody to SERCA1. The following day, protein G-magnetic beads (Millipore) were added, and the mixture was incubated for 1 h in the same condition. Beads were recovered and extensively washed with the lysis buffer and finally aspirated to dryness.

**Aequorin Ca2+ Measurements**—HEK293 cells co-transfected with the cytosolic Ca2+ probe aequorin (cytAEQ) and with empty, WT, and R164H SERCA1-expressing vectors in a 1:1 ratio were preincubated for 1–3 h with 5 µM coelenterazine. Measurements and calibration of aequorin signal were performed as described previously (18). Data are reported as means ± S.D. Statistical differences were evaluated by Student’s two-tailed t test for unpaired samples. A p value of ≤0.05 was considered statistically significant.

**RESULTS**

**Expression of WT and R164H Mutant SERCA1 Protein in HEK293 Cell**—To develop a cellular model suitable for investigating the role of UPS in PMT, HEK293 cells were transfected with cDNA encoding WT (WT-HEK293) or R164H mutant (R164H-HEK293) SERCA1. Heterologous cellular systems were previously successfully used for the investigation of SERCA1 protein that displayed the same features as those found in the muscle SR (12, 14).

Immunofluorescence analysis showed that in WT-HEK293, SERCA1 localized to the endoplasmic reticulum (ER) as shown by the clear co-localization with calreticulin (Fig. 1A), a well known ER marker (19). The R164H SERCA1 mutant was also correctly targeted to ER (Fig. 1A) (see merging with calreticulin), but the expression level was much lower than that of WT SERCA1.

The reduction of SERCA1 immunoreactivity was fully confirmed by immunoblot analysis of total cell lysate with the antibody to SERCA1 (Fig. 1B). On the other hand, blocking of protein neo-synthesis with cycloheximide showed that the stability of SERCA1 mutant was strongly reduced as compared with WT (Fig. 1C). Altogether, these results indicate that this heterologous expression system faithfully mimics the pattern of SERCA1 expression observed in intact muscle fibers from PMT-affected Chianina cattle (6).

**Proteasome Inhibition with MG132 Lactacystin and Bortezomib Prevents Degradation of R164H SERCA1 Protein in Vitro**—To test the role of UPS in causing a reduced expression of R164H SERCA1 mutant, WT-HEK293 and R164H-HEK293 were incubated with MG132, a well recognized proteasome inhibitor. As control, cells were transfected with the empty vector. Although in WT-HEK293 the immunostaining pattern of SERCA1 was slightly positively affected by MG132 treatment, cells expressing R164H SERCA1 mutant exhibited a striking increase of immunoreactivity to SERCA1 after treatment with MG132 (Fig. 2). As expected, no signal of SERCA1 was detected in both treated and untreated cells transfected with the empty vector.

Results obtained by immunofluorescence were paralleled by those obtained by immunoblot analysis of total cell lysate with the same antibody (Fig. 3A). The expression levels of the SERCA1 mutant protein were significantly increased in MG132-treated cells. The graph in the lower part of Fig. 3A shows the average values of SERCA1 expression determined by densitometric analyses of independent immunoblotting experiments. Values, depicted as the percentage of SERCA1 protein content in untreated cells expressing the WT form, showed that after MG132 treatment, the WT protein expression was virtually unaffected, whereas that of the R164H mutant increased, reaching levels comparable with those of WT.

To strengthen the hypothesis that UPS is involved in the reduced expression of R164H SERCA1 mutant, WT-HEK293 and R164H-HEK293 were incubated with other highly specific proteasome inhibitors, lactacystin and bortezomib (Fig. 3B). Immunoblot analysis of total cell lysate with antibodies to SERCA1 confirmed that the expression level of the mutant increased after treatment with the different inhibitors. Taken together, these results strongly support the hypothesis that SERCA1 mutant protein is selectively degraded by the UPS.

Further evidence in favor of this suggestion was gained by assaying the degree of ubiquitination of the SERCA1 mutant. The first step of protein degradation by UPS is the conjugation of target protein with multiple copies of the small protein ubiquitin (20). MG132 inhibits UPS by covalently binding to the active site of the β-subunits of 20 S proteasome (21). This inhibitory effect occurs downstream of the ubiquitination step, allowing the accumulation of polyubiquitinated forms of the protein destined to degradation. Therefore, the treatment with MG132 should allow the recovery of mutated SERCA1 in a polyubiquitinated form. To test this hypothesis, WT-HEK293 and R164H-HEK293 cells, treated or not with MG132, were lysed in mild conditions to permit the subsequent immunopre-
Inhibition of Proteasome Rescues Defective SERCA1

cipitation assay. Under the lysis conditions used, SERCA1 was not totally solubilized, but was partially retained in the insoluble fraction obtained after cell lysate centrifugation (Fig. 4A, lanes 6–10). Soluble supernatants (Fig. 4A, lanes 1–5) were used as input to immunoprecipitate SERCA1 with the specific antibody. Recovered immunocomplexes were then subjected to immunoblot analysis with anti-ubiquitin antibody (Fig. 4B). The antibody recognized trace amounts of ubiquitin linked to WT SERCA1, which slightly increased after proteasomal inhibition. On the other hand, although a remarkable amount of mutant SERCA1 was retained in the insoluble fraction (Fig. 4A), immunoprecipitates from R164H-HEK293 treated with MG132 showed a large increase in polyubiquitination of R164H SERCA1 mutant, as hypothesized.

Qualitative Recovery of R164H Mutant SERCA1 Protein after Proteasome Inhibition in Vitro—These results clearly demonstrate that blockade of UPS activity in R164H-HEK293 causes accumulation of SERCA1 protein. In a previous work, using SR membrane from PMT-affected Chianina muscles, we demonstrated that the SERCA1 mutant displayed functional properties indistinguishable from those of WT SERCA1 (6). Therefore, we hypothesized that R164H SERCA1 mutant rescued by treatment with MG132 should exhibit functional properties similar to those of WT SERCA1. To test this prediction, Ca\(^{2+}\)-ATPase activity was measured, using microsomal membrane fractions isolated from transfected HEK293 cells (14).

As shown in Fig. 5A, microsomes obtained from R164H-HEK293 displayed a Ca\(^{2+}\)-ATPase activity much lower than that of microsomes from WT-HEK293. Incubation with proteasome inhibitor led to an increase in Ca\(^{2+}\)-ATPase activity, which correlated with the increased expression of mutant SERCA1 within ER membranes (Fig. 5B).

To further investigate the functional effect of rescuing mutated SERCA1 by proteasome inhibitor treatment, the ability of either WT or R164H SERCA1 to counteract cytosolic Ca\(^{2+}\) transients induced by cell stimulation was monitored in
HEK293 cells, before and after MG132 treatment. Cells were co-transfected with the cytosolic Ca\(^{2+}\)/H\(\text{11001}\)-sensitive photoprotein aequorin (18) and then stimulated with carbachol, an inositol-1,4,5-generating agonist that induces an increase in the cytoplasmic Ca\(^{2+}\)/H\(\text{11001}\) concentration by causing Ca\(^{2+}\)/H\(\text{11001}\) release from the ER as well as Ca\(^{2+}\)/H\(\text{11001}\) influx across the plasma membrane. Fig. 6A shows the Ca\(^{2+}\)/H\(\text{11001}\) response in untreated cells. The lower peak amplitude and the faster decay kinetics in WT-HEK293 clearly reflected the greater density of the Ca\(^{2+}\)/H\(\text{11001}\) pump as compared with native HEK293 cells (CytAEQ). It is noteworthy that the height of the Ca\(^{2+}\)/H\(\text{11001}\) transient peak was not affected by the overexpression of R164H SERCA1, but the kinetics of the declining phase of the Ca\(^{2+}\)/H\(\text{11001}\) traces in the initial phase immediately after the peak (Fig. 6A) was faster than in native HEK293, and very similar to that of WT-HEK293. Because the declining phase of the Ca\(^{2+}\)/H\(\text{11001}\) transients is an important parameter of Ca\(^{2+}\)/H\(\text{11001}\) handling, its shape confirms that the mutated SERCA1 pump is active. The R164H SERCA1-overexpressing cells are less efficient with respect to WT-HEK293 in counteracting the peak of the Ca\(^{2+}\)/H\(\text{11001}\) concentration transient induced by stimulation, but they restore Ca\(^{2+}\)/H\(\text{11001}\) basal levels faster than native HEK.

These data confirm that the introduction of R164H mutation does not abolish pump activity, in agreement with the experiments where the Ca\(^{2+}\) -ATPase activity was measured in microsomes (Fig. 5A), and suggest that impaired ability to counteract the peak transient depends on the lower amount of SERCA pump expression, as demonstrated by immunoblotting data (Fig. 5B). Thus, to verify whether the recovery of SERCA levels could re-establish Ca\(^{2+}\)/H\(\text{11001}\) extrusion ability in intact cells, cytosolic Ca\(^{2+}\)/H\(\text{11001}\) transients were monitored in cells incubated with MG132 (Fig. 6, C and D). The traces clearly show that the Ca\(^{2+}\)-pumping ability of R164H-HEK293 is indistinguishable from that of WT-HEK293, and is drastically increased with respect to native HEK293. Taken together, these results demonstrate that
the physiological Ca\textsuperscript{2+} homeostasis in cells expressing mutated SERCA1 can be restored by blocking the UPS.

**Electron Microscopy of PMT-affected Muscles**—We have previously demonstrated by immunofluorescence and immunoblotting methods that reduction of mutant SERCA1 in SR membrane of PMT-affected cattle was selective, as both longitudinal SR and junctional SR were not depleted of their main protein components (6). Ultrastructural studies of affected muscle fibers and control sample confirmed that the general structure of muscle fibers was normal (Fig. 7). Triads, although infrequent in both control and pathological muscles, were normal in size and distribution.

**Quantitative and Qualitative Rescue of R164H SERCA1 Protein after Proteasome Inhibition in ex Vivo Experiments**—Rescue of R164H SERCA1 by MG132 was further investigated in adult muscle fibers from PMT-affected Chianina cow. Small bundles of fibers from freshly isolated biopsies were maintained under tissue culture conditions as explant, in the absence and in the presence of MG132 (16). At the end of the incubation period, a crude post-mitochondrial membrane fraction, enriched in content of SR membranes, was isolated (6). Fig. 8A shows the immunoblot analysis with antibody to SERCA1 of subfractions obtained with this method. In agreement with in vitro results obtained with transfected cells, in these ex vivo experiments, the treatment with MG132 also efficiently increased R164H SERCA1 mutant expression. The rescued protein was detected only in the SR microsomal fraction, being almost totally absent from both the soluble cytoplasm and the myofibrillar fraction, in agreement with previous results (6, 15).

As anticipated, the SR-enriched membrane fraction obtained from MG132-treated muscle fibers displayed a large increase in maximal Ca\textsuperscript{2+}-ATPase activity, measured at optimum pCa (Fig. 8B).

**DISCUSSION**

In a previous work, we reported that a missense mutation in ATP2A1 gene, leading to an R164H substitution in the SERCA1 protein (2), underlies Chianina cattle PMT. The skeletal muscles of affected cattle are characterized by a selective reduction in the level of R164H SERCA1 protein in SR membranes (6). Because the mutation does not affect the transcription of ATP2A1 gene, we put forward the hypothesis that the reduction of mutated SERCA1 protein was due to degradation by the UPS. In this study, using a combined experimental approach involving both a heterologous HEK293 cellular model overexpressing the R164H mutant of SERCA1 and biopsies from cattle pathological muscle, we provide unequivocal evidence that the UPS is involved in degradation of mutated SERCA1 protein in PMT-affected Chianina muscles.
By means of the widely used proteasome inhibitors MG132, lactacystin, and bortezomib, known to block UPS downstream to the ubiquitination step, we show that R164H mutant undergoes ubiquitination and that, after blocking proteasome, R164H SERCA1 protein accumulates in HEK293 cells at levels almost comparable with those of cells expressing WT SERCA1. Furthermore, we show that the R164H mutation does not interfere with the proper localization of SERCA1 pump because the rescued protein is correctly targeted to ER. These conclusions are further strengthened by results obtained with adult skeletal muscle fibers from a biopsy from a PMT subject, demonstrating that *ex vivo* treatment with MG132 leads to the selective accumulation of rescued R164H SERCA1 mutant in SR membranes.

In previous work, in which functional properties of R164H bovine SERCA1 protein were compared with those of rabbit and human homologs (22), we hypothesized that the SERCA1 protein, although mutated, was fully functionally active and that the prolonged Ca\textsuperscript{2+} transients, responsible for muscle contractures in PMT-affected cattle, were likely due to the reduced amount of SERCA1 protein pumps in the SR membrane. In support of this hypothesis, Ca\textsuperscript{2+}-ATPase activity in microsomes isolated from transfected HEK293 cells, after treatment

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**FIGURE 6. Monitoring of cytosolic Ca\textsuperscript{2+} concentration in HEK293 cells.** The plasmid expressing the cytosolic Ca\textsuperscript{2+}-sensitive photoprotein aequorin was co-transfected in HEK293 cells with either WT or R164H mutant (R164H) SERCA1 cDNAs or the empty vector (CytAEQ). A and B, co-transfected cells were treated with the vehicle alone (DMSO). C and D, co-transfected cells were treated with the proteasome inhibitor MG132 dissolved in DMSO (indicated as + MG132). Cytosolic Ca\textsuperscript{2+} transients (A and C) were measured after agonist stimulation (500 μM carbachol). The histograms in B and D show the means ± S.D. of Ca\textsuperscript{2+} transient peaks from six independent experiments.
Recently, we provided the crystal structure of the bovine SERCA1, crystallized in the E1 conformation (Protein Data Bank (PDB) accession code 3TLM) (22). The bovine molecular model has proved to be very similar to that of the rabbit enzyme (25, 26), as expected by the high degree of similarity of the amino acid sequence. The R164H substitution (Fig. 9) occurs in the A domain (27) at the N terminus of SERCA1 protein. A detail of the bovine SERCA1 structure centered on the Arg\(^{164}\) (22) has shown that the long side chain of positively charged Arg could be counterbalanced by the negatively charged side chain of Glu\(^2\) and in addition could form extensive interactions with four surrounding residues: Ile\(^{165}\), Thr\(^{191}\), Lys\(^6\), and His\(^6\).

So it is conceivable that Arg\(^{164}\), acting as an anchor point, could be central to proper folding. The substitution of the side chain of the Arg with the imidazole ring of the His residue, which is smaller and which at physiologic pH can be neutral, could destabilize interactions with surrounding residues. The resultant protein, although functional, could be affected in the proper folding at its N terminus, a region described as critical for the correct folding and for maintenance of the three-dimensional structure of the protein (28). In a study on the possible role of the N-terminal region of Ca\(^{2+}\)-ATPase SERCA1, it has been demonstrated that a small structural defect in this domain could be fundamental in inducing an accelerated degradation (28).

Our results, showing that R164H mutation generates a SERCA1 protein most likely corrupted in proper folding, allow classification of cattle PMT into the family of unfolded protein diseases, an extremely heterogeneous and growing group of genetic disorders in which the pathogenic hallmark is the premature degradation or aggregation of the misfolded gene product (29). Moreover, the relevance of these experiments to human pathology is due to the similarity between Chianina cattle PMT and human Brody disease, a rare genetic disorder due to a defect of ATP2A1 gene. Pseudomyotonia is the most important clinical feature, and a diminished SR Ca\(^{2+}\)-ATPase activity is the major biochemical finding (30). However, the mechanism underlying this functional impairment is not fully clarified. At least in some patients, it is clear that ATP2A1 mutations lead to a reduced expression of SERCA1 protein (31). This subgroup of patients is very similar biochemically and phenotypically to PMT-affected Chianina cattle.

At present, no specific therapy exists for human Brody disease. The main finding of this study is that proteasomal inhibition functionally rescues the mutated SERCA1 in a cell model as well as in muscle fibers from PMT-affected animals. This is a very important result because it paves the way to a possible therapeutical approach \textit{in vivo}. This perspective seems to be even more plausible because we confirm here, using electron microscopy, that the ultrastructure of SR is well preserved in muscle specimens from PMT-affected animals, in full agreement with findings in muscle fibers from human Brody patients (31). Our results suggest that acting on the degradative pathway to avoid the premature disposal of mutated SERCA1, might have beneficial effects at least in the specific population of patients in which a reduced expression of SERCA1 has been documented.
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Inhibition of Proteasome Rescues Defective SERCA1

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