A Defective SERCA1 Protein Is Responsible for Congenital Pseudomyotonia in Chianina Cattle

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Recently, a muscular disorder defined as “congenital pseudomyotonia” was described in Chianina cattle, one of the most important Italian cattle breeds for quality meat and leather. The clinical phenotype of this disease is characterized by an exercise-induced muscle contracture that prevents animals from performing muscular activities. On the basis of clinical symptoms, Chianina pseudomyotonia appeared related to human Brody’s disease, a rare inherited disorder of skeletal muscle function that results from a sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA1) deficiency caused by a defect in the *ATP2A1* gene that encodes SERCA1. SERCA1 is involved in transporting calcium from the cytosol to the lumen of the sarcoplasmic reticulum. Recently, we identified the genetic defect underlying Chianina cattle pseudomyotonia. A missense mutation in exon 6 of the *ATP2A1* gene, leading to an R164H substitution in the SERCA1 protein, was found. In this study, we provide biochemical evidence for a selective deficiency in SERCA1 protein levels in sarcoplasmic reticulum membranes from affected muscles, although mRNA levels are unaffected. The reduction of SERCA1 levels accounts for the reduced Ca^{2+} -ATPase activity without any significant change in Ca^{2+} -dependency. The loss of SERCA1 is not compensated for by the expression of the SERCA2 isoform. We believe that Chianina cattle pseudomyotonia might, therefore, be the true counterpart of human Brody’s disease, and that bovine species

might be used as a suitable animal model. (*Am J Pathol* 2009, 174:565–573; DOI: 10.2353/ajpath.2009.080659)

In skeletal muscle fibers, the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) is responsible for transporting Ca^{2+} from the cytosol into the sarcoplasmic reticulum (SR) lumen. SERCA is the main protein component of nonjunctional SR,¹ comprising almost 90% of the total proteins.² Three isoforms of SERCA proteins are differentially expressed by three genes.³ Additional isoforms are expressed by alternative splicing, each gene encoding different isoforms. The SERCA1 isoform is expressed in fast-twitch (type 2) skeletal muscle. Cardiac and slow-twitch skeletal muscle (type 1) expressed a unique SERCA2a isoform, distinct from SERCA1 isoform, but with 84% sequence homology.³ The SERCA2b isoform is found predominantly in smooth muscle and non-muscle cells, together with SERCA3.⁴

A congenital muscular disorder has been described recently in Chianina cattle.⁵ Chianina is one of the most important Italian breeds for meat quality (Fiorentina steak) and leather (used in high design easy-chair manufacture). Clinically, the disorder is mainly characterized by muscle contracture that prevents animals from performing muscular activities. Animals experience the muscle contracture when stimulated to move faster than a simple walk at a slow pace, after transport stress, or when startled. After a few minutes, the stiffness disappears⁵ and the animals regain the ability to move. On the basis of clinical findings, Chianina cattle muscular disorder was defined as “congenital pseudomyotonia” (PMT),⁵ and authors pointed out that the clinical picture closely resembles human Brody’s disease.⁵

In 1969, Brody first described in a human patient a muscular disorder characterized by an “impairment of

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muscle relaxation."⁶ So far, human Brody's disease is known as a rare inherited disorder of skeletal muscle due to an SR Ca^{2+} -ATPase deficiency, resulting from a defect of *ATP2A1* gene coding for SERCA1 isoform. The nature of the defect underlying SR Ca^{2+} -ATPase deficiency is still controversial. Some authors attributed the SR Ca^{2+} -ATPase deficiency to a reduced Ca^{2+} -ATPase expression in pathological muscles.^{7,8} Other groups showed that the SR Ca^{2+} -ATPase deficiency did not correlate with a reduction in Ca^{2+} -ATPase protein content.^{9,10} Human Brody's disease was also genetically heterogeneous. Mutations have been reported in the *ATP2A1* gene of Brody myopathy-affected members families leading to premature stop codons¹¹ or to a substitution of Pro789 to Leu.¹²

Recently, we have fully characterized the genetic defect underlying Chianina PMT by DNA sequencing 15 PMT-affected calves.¹³ In all cases examined, a single point mutation (c.491G>A) in exon 6 of the *ATP2A1* gene, leading to an Arg 164 His substitution in the SERCA1 protein, was observed.¹³ All PMT-affected animals were found to be homozygous.¹³ On the basis of these results and with the aim of confirming the similarity of Chianina cattle PMT with human Brody's disease, here we report a biochemical characterization of activity and content of the mutated Ca^{2+} -ATPase SERCA1 in SR-enriched microsomal fractions extracted from pathological muscles.

Materials and Methods

Materials

A23187 Ca^{2+} -ionophore was purchased from Calbiochem (San Diego, CA). All chemicals were analytical grade and were purchased from Sigma-Aldrich.

Case Reports

We investigated four Chianina animals, three males (reported as case 1, case 3, and case 4) and one female (case 2) affected by a muscle function disorder. Chianina specimens were hospitalized at the Veterinary Clinical Department of University of Bologna in compliance with Italian Law (Decreto legge September 27, 1992, no 116). Biopsies of semimembranosus muscle for diagnostic evaluations were performed on animals under local anesthesia. Four semimembranosus muscle samples were collected from normal Chianina animals that were euthanized at a slaughterhouse and used as controls. The existence of glycolytic, mitochondrial, and lipid storage myopathies was excluded by appropriate investigations.⁵

Preparative Procedures

Biopsies from semimembranosus muscle, a representative fast-twitch skeletal muscle,¹⁴ were homogenized in 10 mmol/L HEPES, pH 7.4, 20 mmol/L KCl containing 1 $\mu\text{g/ml}$ leupeptin and 100 $\mu\text{mol/L}$ phenylmethylsulfonyl fluoride, as described.^{15,16,17} The myofibrils were sedi-

mented by centrifugation at $650 \times g$ for 10 minutes at 4°C. The crude SR fraction was obtained from the previous supernatant by ultracentrifugation at $120,000 \times g$ for 90 minutes at 4°C. The final supernatant, representing the soluble sarcoplasm, was saved. Membrane fractions were resuspended in 0.3M/L sucrose, 5 mmol/L imidazole, pH 7.4, containing 1 $\mu\text{g/ml}$ leupeptin and 100 $\mu\text{mol/L}$ phenylmethylsulfonyl fluoride and stored at -80°C. Protein concentration was determined by the method of Lowry et al¹⁸ using bovine serum albumin as standard.

Gel Electrophoresis and Immunoblotting

SDS-polyacrylamide gel electrophoresis (PAGE)¹⁹ and immunoblotting were performed as described.^{16,17} The blots were probed with: (i) mouse monoclonal antibodies to fast myosin heavy chain (MHC, 1:2000, Sigma); sarcalumenin and GP53 splice variant (a SR glycoprotein of 53 kDa, 1: 2000, AffinityBioReagents, Golden CO); SERCA1 (recognizing an epitope between amino acid residues 506 and C-terminus of rabbit fast twitch SR Ca^{2+} -ATPase, 1:2000, Biomol, Plymouth Meeting, PA); SERCA1 (recognizing an epitope between amino acid residues 199 to 505 of rabbit fast twitch SR Ca^{2+} -ATPase, 1:2000, AffinityBioReagents, Golden CO); SERCA2 (1:2000, Biomol, Plymouth Meeting, PA); (ii) rabbit polyclonal antibodies: FKBP-12 (1:2500, AffinityBioReagents, Golden CO); Calsequestrin (CS, 1: 2500, AffinityBioReagents, Golden CO); and (iii) chicken polyclonal antibodies to rabbit fast twitch Ca^{2+} -ATPase (5 $\mu\text{g/ml}$).²⁰

Biochemical Assay

ATPase activity of the microsomal fractions (20 $\mu\text{g/ml}$) was measured by spectrophotometric determination of NADH oxidation coupled to an ATP regenerating system, as previously described.^{20,21} The assay was performed at 37°C in the presence of 2 $\mu\text{g/ml}$ A23187 Ca^{2+} -ionophore at pCa5. For investigating the Ca^{2+} -dependence of ATPase activity, the concentration of free Ca^{2+} was varied from pCa9 to pCa3 using EGTA buffered solutions.²²

Histology, Histochemistry, and Immunohistochemistry

Muscle biopsies of the semimembranosus muscle were taken from congenital PMT-affected Chianina cattle at rest. Muscle samples were frozen in cold isopentane and sections (10 μm) were cut in a cryostat. Sections were incubated with monoclonal primary antibodies (dilution 1:100) to SERCA1 (recognizing an epitope between amino acid residues 506 and C-terminus of rabbit fast twitch SR Ca^{2+} -ATPase; Biomol, Plymouth Meeting, PA), SERCA2 (Biomol, Plymouth Meeting, PA), and phospholamban (PLB, AffinityBioReagents, Golden CO), followed by incubation with secondary antibody conjugated with tetramethylrhodamine isothiocyanate (Dako, Milano, It-

aly) as described.^{16,17} Confocal microscopy was performed using a Leica TCS-SP2 confocal laser scanning microscope.

Semimembranosus muscle biopsies were taken from the same animals after muscle exercise induced by stress. The exercise test protocol lasted 10 minutes and was performed three times a day for 10 days. Serial cryostat sections were stained for Gomori's trichrome, periodic acid-Schiff, oil red O, succinic dehydrogenase, cytochrome oxidase, and myofibrillar adenosine triphosphatase (m-ATPase), or were immunostained by incubating with polyclonal antibodies (dilution 1:200) to neonatal MHC isoform,²³ followed by incubation with secondary antibody conjugated with peroxidase (Dako, Milano, Italy). The reaction was visualized with the Envision method (Dako).

RNA Preparation and Quantitative Real-Time PCR

Total RNA was isolated from semimembranosus muscles using TRIzol reagent (Invitrogen, Milano, Italy) and trace genomic DNA contamination from total RNA was removed by DNase I treatment (Invitrogen), according to the manufacturer's instructions. The integrity of each of the RNA samples was assessed by electrophoresis (28S:18S ratio) and the concentration was estimated spectrophotometrically. Two micrograms of total RNA from each sample were subjected to random hexamer primed first-strand cDNA synthesis in a volume of 20 μ l using Superscript II reverse transcriptase (Invitrogen), according to manufacturer's instructions. Real-time PCR was performed by the SYBR Green method with an Applied Biosystems 7500 Fast Real Time PCR System as described.²⁴ For comparative evaluation of transcript levels, standard curves using cDNA serial dilutions (from 100 to 0.001 ng) were first analyzed and used to calibrate the relative expression of the gene of interest. Calculations were made using the Applied Biosystems software based on threshold (Ct) values. Real-time PCR amplification was performed in a total volume of 30 μ l, containing 10 or 1 ng of cDNA of each sample, 9 pmol of each primer. Reaction conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15s at 95°C, 1 minute at 60°C, followed by the melting curve protocol to verify the specificity of amplicon generation. All samples were run in triplicate. All values were normalized relative to the expression of glyceraldehydes-3-phosphate dehydrogenase using gene specific primers. Oligonucleotide primers used were: SERCA1 5'-GCACTCCAAGACCACAGAAGA-3' (sense), 5'-GAGAAGGATCCGCACCAG-3' (antisense); glyceraldehydes-3-phosphate dehydrogenase 5'-GGT-CACCAGGGCTGCTTTTA-3' (sense), 5'-GAAGATGGT-GATGGCCTTTCC-3' (antisense).

Statistical Analysis

Data are expressed as means $\pm$ SD. One-way Anova computation test was used to analyze data. All procedures were taken from GraphPad Prism4.

Results

Histological and Immunohistochemical Analysis of Muscle Biopsies from Affected Chianina Cattle at Rest and after Muscle Exercise Protocol

To investigate Chianina cattle PMT, we first performed routinely morphological (Gomori trichrome) and histochemical (periodic acid-Schiff, cytochrome oxidase, succinic dehydrogenase, m-ATPase) analysis on semimembranosus muscle biopsy sections. When animals were kept at rest or moved at a slow pace, we did not observe any contracture.⁵ Furthermore, trichromic staining of cryostat sections (Figure 1A) showed normal fiber diameter, and no evidence of muscle damage. Also periodic acid-Schiff, cytochrome oxidase, succinic dehydrogenase, m-ATPase, and oil red stainings (data not shown) did not show morphological alterations, additionally indicating that normal fiber type distribution was maintained. When trichromic staining was performed on muscle biopsy obtained from animals that had performed the exercise test protocol, dark giant fibers with large areas of necrosis were observed (Figure 1B). Immunostaining with anti-neonatal MHC antibody (Figure 1C) revealed the presence of small and medium size positive fibers at the periphery of the necrosis areas (Figure 1C inset), likely corresponding to activated satellite cells. The degree of muscle damage and regeneration observed in Chianina muscles varied depending on subject's sensitiveness to the exercise protocol test.

Immunofluorescence Microscopy, Biochemical Analysis of SR Membranes, and Measurement of the Level of Expression of mRNA for SERCA1

In a recent work,¹³ we have reported that homozygous, PMT-affected Chianina animals carried a single point mutation within exon 6 of *ATP2A1* gene, coding for SERCA1, leading to an Arg 164 His substitution.¹³

To investigate the effect of this mutation on the level of expression of SERCA1 in affected muscles, a simultaneous comparison of transverse cryosections from control (Figure 2A, panel a) and pathological muscles (Figure 2A, panels b, c, d) was performed, using a monoclonal antibody to SERCA1 isoform. Results showed that most of the fibers were labeled (Figure 2A) and only a small number of fibers was unstained with this antibody, likely representing type 1 fibers. To confirm this interpretation, serial cross sections (Figure 2B) were incubated with antibodies against SERCA1 (Figure 2B, panel a), SERCA2 (Figure 2B, panel b), and against SERCA2-regulatory protein PLB,²⁵ another undisputed marker of type 1 fibers (Figure 2B, panel c). Results showed that semimembranosus muscle contained only two types of fibers, expressing in a mutually exclusive manner, either SERCA1 (Figure 2B, panel a) or SERCA2 and PLB (Figure 2B, panels b and c). No difference was observed between control and pathological muscles (not shown).

Figure 1. Histological and immunohistochemical stainings on semimembranosus muscle sections from congenital PMT-affected Chianina animals under resting conditions (**A**) and after a muscle exercise protocol (**B, C**). Transversal sections from muscle biopsies taken before (**A**) and at the end of muscle exercise test protocol (**B**) were stained with Gomori's trichrome staining. In panel **B**, the dark giant fibers with a large area of necrosis are indicated with **asterisks** and are shown in more detail in the **inset**. **C**: Transversal muscle sections were immunolabeled with anti-neonatal MHC isoform antibodies. Regenerating fibers at different stages of development, positive to anti-neonatal MHC antibodies, are indicated by **arrows**. In the **inset**, two small immature fibers surrounding a degenerative fiber are shown. Scale bars = 50 μ m.

These results are in full agreement with the knowledge that semimembranosus muscle is predominantly fast-twitch muscle, containing only 8% to 10% of slow-twitch type 1 fibers, based on the MHC isoforms composition.¹⁴

By comparison with normal muscle (Figure 2A, panel a), PMT-affected muscle specimens (Figure 2A, panels

Figure 2. Confocal microscopy of semimembranosus muscle cryosections from control and congenital PMT-affected Chianina calves. **A**: Transverse sections from control (**panel a**) and PMT-affected calves (**panel b** case 3; **panel c**, case 1; **panel d**, case 2), were immunolabeled with monoclonal antibodies to SERCA1 (recognizing an epitope located in COOH-terminus). In pathological muscles, a reduction of immunoreactivity was revealed when compared with normal muscle. Scale bar = 150 μ m. **B**: Serial cross-sections from a pathological muscle (case 3) were immunostained with antibodies to SERCA1 (**panel a**), SERCA2 (**panel b**), and PLB (**panel c**). PLB, the SERCA2 isoform regulatory protein, is uniquely expressed in slow-twitch fibers, in which it colocalizes with the corresponding Ca^{2+} -ATPase isoform. Scale bar = 50 μ m.

b, c, d) revealed a reduction of immunoreactivity to SERCA1. The degree of reduction was from mild (Figure 2A, panel b, case 3), to medium (Figure 2A, panel c, case 1), and appeared severe in one sample (Figure 2A, panel d, case 2).

To investigate the reduction of SERCA1 immunoreactivity, a crude microsomal fraction enriched in content of SR membranes^{16,17} was isolated by differential centrifugation from muscle biopsies obtained from control and pathological muscles (see Materials and Methods). Figure 3A shows an immunoblot analysis of fractions obtained with this method with antibodies to SERCA1 (recognizing an epitope located in COOH-terminus) and MHC. In both control and pathological samples, SERCA1

Figure 3. Distribution of SERCA1 protein within muscle subfractions (**A**) and expression levels of SERCA1 (**B**) and SERCA2 (**C**) proteins in normal and congenital PMT-affected Chianina calves. **A:** Muscle homogenates from control (lanes 1, 2, 3) and pathological samples (lanes 4, 5, 6 case 3; lanes 7, 8, 9 case 4) were subfractionated as described in Materials and Methods, and an equal quantity (3 μ g/lane) of protein was separated by 5% to 10% SDS-PAGE, followed by Western blot analysis with the specific monoclonal antibodies to fast MHC and SERCA1 (recognizing an epitope located in COOH-terminus), as indicated. Lanes 1, 4, 7: crude microsomal fraction enriched in content of SR membranes. Lanes 2, 5, 8: soluble supernatant. Lanes 3, 6, 9: myofibrillar fraction. **B, C:** Crude SR microsomal fractions from controls (lanes 1 to 4) and PMT-affected Chianina cattle (lanes 5 to 8), were separated by 5% to 10% SDS-PAGE and blotted onto nitrocellulose (protein loading was 3 μ g/lane). The blots were incubated with one polyclonal and two different monoclonal antibodies to SERCA1 isoform as indicated (**B**) or with monoclonal antibodies to SERCA2 isoform (**C**). Lane 5: case 1; lane 6: case 2; lane 7: case 3; lane 8: case 4. SERCA1 band is faintly detected in case 1 only with the polyclonal antibodies and almost undetectable in case 2. In cases 3 and 4 the intensity of staining is reduced.

protein was detected only in the microsomal fraction (Figure 3A, lanes 1, 4, 7), being totally absent from the soluble cytoplasm (Figure 3A, lanes 2, 5, 8), as well as from the myofibrillar fraction (Figure 3A, lanes 3, 6, 9), as expected.^{15,16,17} SERCA1 appeared reduced in fractions from pathological muscles (Figure 3A, lanes 4, 7). Myosin contamination of the microsomal fraction was negligible, as detected by immunoblot with antibodies to MHC (Figure 3A, lanes 1, 4, 7). Equal SR microsomal fractions from affected animals (Figure 3B, lanes 5, 6, 7, 8) and by comparison from control muscles (Figure 3B, lanes 1, 2, 3, 4), probed with the anti-SERCA1 monoclonal antibodies, established a reduction of SERCA1 protein expression in all pathological samples examined (Figure 3B, lanes 5, 6, 7, 8). Identical results were obtained on incubation of microsomal fractions with both a different monoclonal antibody to SERCA1 (recognizing an epitope located in NH2-terminus, Figure 3B) and with a polyclonal antibody, against Ca^{2+} -ATPase SERCA1 isoform (Figure 3B). The decreased content of SERCA1 in the microsomal fraction could not be explained by a different pattern of distribution between fractions, since Figure 3A shows that SERCA1 was detectable only in the SR fraction also in the pathological muscles. Figure 3B clearly shows also that affected muscles (Figure 3B, lanes 5, 6, 7, 8) were heterogeneous with respect to the content of SERCA1. In particular, the SERCA1 protein band is faintly detected in

Figure 4. Immunodetection of SR protein markers in the microsomal fraction deriving from SR. **A, B:** Crude SR microsomal fractions from controls (lanes 1 to 4) and affected Chianina calves (lanes 5 to 8) were separated by 5% to 10% SDS-PAGE and blotted onto nitrocellulose. The blots were incubated with the indicated antibodies. Lane 5: case 1; lane 6: case 2; lane 7: case 3; lane 8: case 4.

case 1 (lane 5), being virtually absent in case 2 (lane 6). On the other hand, the decrease in the content of SERCA1 was not counterbalanced by a corresponding increase of SERCA2 isoform. Figure 3C demonstrates that no differences exist in SERCA2 content between normal (Figure 3C, lanes 1 to 4) and affected animals (Figure 3C, lanes 5 to 8).

SR fractions from pathological and control muscles were also probed with specific antibodies against protein markers of junctional (FKBP12 and CS, Figure 4A) and nonjunctional (160 kDa sarcalumenin and 53 kDa glycoprotein)²⁶ (Figure 4B) SR membranes. Figure 4 shows that SR fractions obtained from pathological muscles did not differ from control muscles for the content of either FKBP12 or sarcalumenin and 53-kDa glycoprotein, indicating that only SERCA1 was selectively affected. Also using a polyclonal antibody that recognizes both isoforms of CS, no differences in the expression of the CS skeletal isoform (Figure 4A) were found between control (Figure 4A, lanes 1 to 4) and pathological (Figure 4A, lanes 5 to 8) muscles. A protein band of about 55 kDa corresponding to the slow/cardiac CS isoform was detected in SR membranes obtained from pathological case 2 (Figure 4A, lane 6), while being present only in trace amount in pathological case 1 (Figure 4A, lane 5) and totally absent from case 3 and case 4 (lanes 7 and 8). No evidence whatsoever for the presence of this protein was found in control samples (Figure 4A, lanes 1 to 4).

Altogether, our results demonstrate that the decrease in immunoreactivity observed by immunofluorescence is due to a selective reduction of the levels of expression of SERCA1 protein. To investigate the possibility that the reduced content of SERCA1 displayed by SR membranes might be due to a reduced *ATP2A1* transcriptional activity, we performed real-time PCR experiments (Figure 5). Our data demonstrate that SERCA1 mRNA expression levels were comparable in all pathological samples and equal to the controls (Figure 5).

Figure 5. Expression levels of SERCA1 mRNA in control and PMT-affected Chianina muscles. SERCA1 mRNA levels were quantified by real-time RT-PCR. Data are reported as the relative expression with respect to control muscle. Control data were obtained from different mRNA extractions from three unaffected Chianina specimens. The values are the mean $\pm$ SD ($n = 6$). Mean values were found not to be statistically different.

Measurements of SR Ca^{2+} -ATPase Activity

The Ca^{2+} -ATPase activity of SR membranes was measured at optimum pCa (pCa5), in the presence of Ca^{2+} -ionophore A23187 (Figure 6A). Membranes from control samples displayed an average Ca^{2+} -ATPase activity of 2.21 ± 0.6 SD $\mu\text{mol}/\text{min}/\text{mg}$ prot (mean value of five determinations performed on each four different control

Figure 6. Ca^{2+} -ATPase activity and Ca^{2+} -dependence of SR microsomal fractions from controls and PMT-affected Chianina cattle. **A:** The Ca^{2+} -ATPase activity was determined by a spectrophotometric enzyme-coupled assay at optimum pCa (pCa 5) in the presence of ionophore A23187 as described in Materials and Methods. Control values were obtained from different preparations of microsomes obtained from four unaffected Chianina specimens. Data are expressed as percentage of control value and are the mean $\pm$ SD. **B:** The Ca^{2+} -ATPase activity was determined at various concentrations of free Ca^{2+} . Data are expressed as percentage of control values and are the mean $\pm$ SD. Symbols: circle = control animals (from different preparations of microsomes from four unaffected Chianina specimens); square = case 3 pathological animal; triangle = case 4.

samples). This value is lower than that obtained for rabbit,²¹ but in the range of those obtained for human²⁰ muscle. The Ca^{2+} -ATPase activity of SR fractions from cattle pathological muscle was 0.17 ± 0.043 SD $\mu\text{mol}/\text{min}/\text{mg}$ prot (average of five determinations on the same fraction) for case 1, 0.70 ± 0.053 SD $\mu\text{mol}/\text{min}/\text{mg}$ prot (average of 13 determinations on the same fraction) for case 4, and 0.95 ± 0.14 SD $\mu\text{mol}/\text{min}/\text{mg}$ prot (average of 17 determinations on the same fraction) for case 3 respectively. The Ca^{2+} -ATPase activity was almost undetectable in the case 2 (Figure 6A). These results nicely correlate with the reduction of SERCA1 demonstrated by immunoblotting (Figure 3B). On the other hand, the Ca^{2+} -dependence of Ca^{2+} -ATPase activity (Figure 6B) resulted similar for pathological and control samples (K_{50} calculated by Hill plot analysis was around 2.0×10^{-7}).

Discussion

In previous work, Testoni and co-workers⁵ described for the first time in Chianina cattle a muscle function disorder called PMT, characterized by muscle stiffness.⁵ The impaired muscle relaxation exhibited by PMT calves closely resembled the symptoms of patients affected by Brody's disease.⁵ Even though a search for mutations in *ATP2A1* gene revealed genetic heterogeneity,^{11,12} the mechanism underlying human pathology was found to be an abnormally low rate of Ca^{2+} uptake into the SR, resulting either from a reduced expression of SERCA1 protein^{7,8} or from a reduced activity of the normally expressed enzyme.^{9,10}

Similarities between clinical phenotypes of Brody's disease and Chianina PMT⁵ prompted us to suppose that the delayed muscles relaxation observed in Chianina cattle might be the consequence of prolonged elevation in cytoplasmic free Ca^{2+} concentration, and that a genetic defect of SERCA1 might also underlies Chianina PMT. This hypothesis turned out to be correct. By DNA sequencing of PMT-affected calves we have provided evidence of a missense mutation (c. 491G>A) in exon 6 of *ATP2A1* gene.¹³ The mutation leads to an Arg 164 His substitution in a highly conserved region of SERCA1 protein. Affected Chianina calves were found to be homozygous, whereas carriers, in which any clinical sign of the PMT has been described, were heterozygous.

In this paper, we provide for the first time direct biochemical evidence of SERCA1 protein deficiency in skeletal muscles of PMT-affected Chianina cattle. As in human Brody's disease, we also found that the Ca^{2+} -ATPase activity of isolated SR membranes was markedly reduced, without any significant change in Ca^{2+} -dependency.

The reduced density of SERCA1 in SR membranes was clearly demonstrated by immunofluorescence analysis of muscle cryosections (Figure 2A), as well as by immunoblot analysis of SR membranes (Figure 3B). In immunoblotting experiments, three different anti-SERCA1 antibodies were used (Figure 3B). It is therefore unlikely that the observed reduction in protein density might be the consequence of an altered antigenicity. Although expressed at lower levels, the association of SERCA1 to

the SR is preserved in pathological muscles, being the protein totally absent in postmicrosomal supernatant and in myofibrillar fraction (Figure 3A). In addition, the loss of SERCA1 protein was never compensated by the expression of slow-twitch muscle isoform SERCA2 (Figure 3C), in accordance with results already described for human muscle cultures.¹⁰ In our samples, the relative contribution of SERCA2 isoform, accounted for by the percentage of slow fibers of semimembranosus muscle, resulted as comparable in normal and pathological muscles (Figure 3C), clearly indicating that type 2 fibers were selectively affected.

The decrease in Ca^{2+} -ATPase activity (Figure 6A), ranging from 7% to 40% of the control values, but the maintenance of Ca^{2+} -dependence (Figure 6B), consistently correlated with the decrease in SR SERCA1 protein level in cattle pathological muscles. These data are in close agreement with measurements of Ca^{2+} -ATPase activity obtained for human microsomal SR fractions from Brody's patients, varying from 2% to 30% of controls.¹⁰

Using electron microscopy techniques, Karpati et al⁷ showed that in muscle biopsies from patients affected by Brody's disease, triads as well as the SR longitudinal component were normal in size and distribution. In accordance with these results, we found that in SR muscle fractions from affected animals, both longitudinal and junctional portions of SR are not depleted of their main protein components (Figure 4, A and B).

In pathological samples, an additional isoform of CS, corresponding to the cardiac/slow isoform, was detected (Figure 4A). The amount of this CS isoform correlated with the severity of the disease. The most likely explanation is that the expression of slow/cardiac CS in pathological samples (Figure 4A), reflects the ongoing regeneration process. In case 2, in which the pathology seems more severe, transport stress conditions may have induced muscle damage similar to that obtained after exercise protocol.

Overall, our results clearly demonstrate that pathological muscles are characterized by a selective reduction in the level of expression of SERCA1 protein, which accounts for the reduced Ca^{2+} -ATPase activity (Figure 6A). The question is: why is the level of expression reduced, since the point mutation did not result in any stop codon, as is the case for some Brody's genotypes,^{11,12} and given also that we failed to detect any reduction of mRNA expression for SERCA1 (Figure 5)?

It is possible that the resulting defective protein could be affected in translation or have an enhanced susceptibility to the protein degradation via ubiquitin-proteasomal and autophagic-lysosomal pathways,²⁷ before being embedded into SR bilayer. In studies of Ca^{2+} -ATPase mutagenesis, MacLennan and co-workers experimentally created several mutations affecting both expression and stability or activity of Ca^{2+} -ATPase.^{28,29} Some Ca^{2+} -ATPase mutants, although stably integrated into membrane, are expressed at low levels in cell lines.³⁰ Moreover, SERCA2b isoform mutations are responsible for human Darier's disease, an autosomal dominant genetic skin disease referred as "keratosis follicularis." The amino acid sequence of SERCA2b is highly homologous to that

of SERCA1. Over 100 mutations have been described in Darier's disease patients, including substitution of amino acid residues.³¹ Several studies reported that the skin pathology is associated with a reduction in expression and activity of SERCA2b,³² and that SERCA2b mutants showed low protein expression and enhanced proteasome-mediated protein degradation.³³ A nucleotide substitution, leading to Arg 164 Ser missense mutation in the A-domain of SERCA2b protein has been found in a Hungarian patient with Darier's disease.³⁴ This amino acid change occurs at the same position to that found in our Chianina samples.

On the basis of our results, a possible pathogenetic sequence can be put forward, as follows. At rest, animals do not show any pathological signs, as demonstrated by histological findings of unstimulated animals (Figure 1A). It is possible that, under resting conditions, the activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and of the plasma membrane Ca^{2+} -ATPase are enough to control Ca^{2+} homeostasis. Muscle stiffness occurred, when "animals are stimulated to move faster than a simple walk at slow pace."⁵ It is conceivable that a larger change of intracellular Ca^{2+} concentration occurs under these conditions. Given the reduced amount of Ca^{2+} -ATPase activity, Ca^{2+} is not pumped back into SR, thereby triggering the contracture. In the long run, this in turn may induce rhabdomyolysis (Figure 1B). The ongoing regeneration clearly indicates activation of satellite cells. In muscle sections obtained after the exercise test, newly regenerating cells surrounding necrotic fibers were clearly detected (Figure 1C). The degree of muscle damage and regeneration found in Chianina muscles varied depending on subject's sensitivity to the exercise protocol test.

During the last 20 years, the counterparts of human pathologies have been found in many domestic mammalian species, including bovine species. On the basis of symptoms⁵ and of genetic¹³ and biochemical confirmations, we concluded that the congenital PMT observed in Chianina cattle, resembles human Brody's disease.

Recently, in the Belgian Blue cattle breed has been described a "congenital muscular dystony."³⁵ The DNA sequencing has revealed a missense mutation resulting in Arg 560 Cys substitution in *ATP2A1* gene and this substitution has been indicated as causative for congenital muscular dystony in the Belgian Blue cattle breed. It has been already demonstrated by site-directed mutagenesis experiments³⁶ that this point mutation causes a strong inhibition of Ca^{2+} -ATPase activity and phosphoenzyme formation due to the principal role of Arg 560 in stabilizing the nucleotide substrate.

As far as we know, Chianina cattle PMT and Belgian Blue cattle congenital muscular dystony³⁵ (two breeds of the same species) are the first pathologies similar to Brody's disease, to be described in non-human mammals, both attributed to a missense mutation in *ATP2A1* gene. In Belgian Blue, the mutation is located within the nucleotide-binding domain while in Chianina the mutation is in the A domain, suggesting that the disease is genetically heterogeneous, as well as in human species. Despite the similarity in gene target defect and clinical picture, affected Belgian Blue cattle don't survive more than

few weeks as a result of respiratory complications. This severe pathology is reminiscent of the effects of SERCA1-null mutant on mouse line.³⁷ The loss of SERCA1 impairs diaphragm function and affected mice died after birth, of respiratory failure.

On the contrary, in Chianina cattle as in human the pathology is not life-threatening and respiratory failure is not a clinical feature: muscle contracture appears only when animals are stimulated to perform intense muscular activities and disappears as soon as the exercise ceases. If Chianina breed animals are allowed to live on their own standards, any contracture crisis can be observed, and although performance is slightly below expectations for Chianina breed, animals reach a satisfactory weight and can be slaughtered. For these reasons we believed that Chianina cattle PMT might be the true counterpart of human Brody's disease and that this bovine species might be used as a suitable animal model.

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