Structural study of skeletal muscle fibres in healthy and pseudomyotonia affected cattle

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S U M M A R Y

Cattle congenital pseudomyotonia (PMT), recognized as naturally occurring animal model of human Brody disease, is an inherited recessive autosomal muscular disorder due to missense mutations in *ATP2A1* gene, encoding sarco(endo)plasmic reticulum Ca^{2+}-ATPase protein, isoform 1 (SERCA1). PMT has been described in the Chianina and Romagnola italian cattle breeds and as a single case in Dutch improved Red and White cross-breed. The genetic defect turned out to be heterogeneous in different cattle breeds, even though clinical symptoms were homogeneous. Skeletal muscles of affected animals are characterized by a selective deficiency of SERCA1 in sarcoplasmic reticulum (SR) membranes. Recently, we provided evidence that in Chianina breed, the ubiquitin proteasome system is responsible for SERCA1 mutant premature disposal, even when the mutation does not affect the catalytic properties of the pump.

Results presented here show that all SERCA1 mutants described until now, although expressed at low level, are correctly targeted to SR membranes. Ultrastructural studies confirm that in pathological muscle fibres, structure, as well as triads, is well preserved.

All together these results suggest that a possible therapeutical approach based on the rescue of the defective protein at SR membranes could be hypothesized. Only fully functionally active missense mutants, whem located at the SR membrane could restore the efficient control of Ca^{2+} homeostasis and prevent the appearance of the pathological signs. Moreover, these data demonstrate the increasing importance of domestic animals as genetic models of human pathologies.

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1. Introduction

The bovine "congenital pseudomyotonia" (PMT) is a muscular disorder clinically characterized by stiffness and progressive impairment of muscle relaxation induced by intense exercise (Testoni et al., 2008). Recently PMT was depicted as the true and unique animal model, although unconventional, of human Brody disease (Brody, 1969). Muscle contractures occur when animals are forced to move fast or are exposed to transport stress. If the exercise is prolonged, the contracture hampers the movement and affected animals consequently fall down. After a few seconds of rest, the stiffness disappears and the animals regain the ability to get up and move. Up to now, PMT pathological condition has been reported in Chianina (Testoni et al., 2008), in Romagnola (Murgiano et al., 2012) italian breeds and as a single case in a Dutch improved Red and White cross-breed calf (Grünberg et al., 2010) .

Cattle PMT is an inherited recessive autosomal disorder, due to a sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) deficiency, resulting from a defect of *ATP2A1* gene, coding for SERCA1 isoform (Drögemüller et al., 2008). In mammals, three genes encode three main SERCA isoforms which are expressed in striated muscles in a tissue-dependent fashion and according to the stage of development (Periasamy and Kalyanasundaram, 2007; Vangheluwe et al., 2009). SERCA1 isoform is expressed in adult fast-twitch type II skeletal muscle fibres. The SERCA2a variant is selectively expressed in slow-twitch type I skeletal muscle fibres and in the heart.

SERCA is the main intrinsic protein component of non-junctional sarcoplasmic reticulum (SR) membranes (Fleischer and Inui, 1989). It consists of three cytoplasmic domains, called Nucleotide-binding (N) Phosphorylation (P) and Actuator (A) domains, 10 transmembrane helices (M domain) and small SR luminal loops (Toyoshima et al., 2000).

The bovine PMT disease has also been found to be genetically heterogeneous. All PMT affected Chianina animals were homozygous for a single point mutation in exon 6 of *ATP2A1* gene.

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The mutation leads to an Arg164His (R164H) substitution in the cytosolic A domain of SERCA1 protein (Drögemüller et al., 2008). Romagnola PMT affected calves were either compound heterozygous or homozygous for *ATP2A1* mutations (Murgiano et al., 2012). Most of the Romagnola pathological cases were compound heterozygous, carrying a single point mutation in exon 6 identical to that of Chianina breed, in addition to two point mutations in exon 8. The mutations in exon 8 lead to a Gly211Val and Gly286Val (G211V/G286V) substitutions in the A domain and in the short luminal loop in the direct proximity of fourth transmembrane domain, respectively. One Romagnola pathological subject did not carry the exon 6 variant, but was homozygous for the two mutations on exon 8. The Dutch improved Red and White cross-breed calf was found to be homozygous for a *ATP2A1* mutation leading an Arg559Cys (R559C) substitution in the highly conserved cytoplasmic N domain of SERCA1 protein (Grünberg et al., 2010).

In spite of genetic heterogeneity, PMT-affected bovine muscles from all breeds examined were characterized by a striking, selective reduction of SERCA1 protein content at SR membrane (Sacchetto et al., 2009; Murgiano et al., 2012; Dorotea et al., 2015). SERCA pump transports two Ca^{2+} ions from the cytoplasm to SR lumen at the expense of the hydrolysis of one ATP molecule, playing a crucial role in muscle relaxation and in the maintenance of resting intracellular Ca^{2+} concentration. As muscle stiffness occurred when animals were stimulated to perform intense muscular activities, we concluded that the reduced amount of SERCA1 protein in the SR membranes of PMT-affected animals was not sufficient to pump back the larger change of intracellular Ca^{2+} concentration occurring under sustained exercise, thereby triggering contractures (Sacchetto et al., 2009).

In the present study, using immunofluorescence and ultrastructural analyses, we investigated the general structure of muscle fibres and the association to SR membranes of mutant SERCA1 protein in pathological muscles from all PMT-affected cattle breeds and by comparison in bovine control healthy muscle.

2. Materials and methods

2.1. Muscle biopsies

Semimembranosus muscle samples were obtained from four Chianina, four Romagnola PMT-affected animals and from the single case of PMT Dutch improved Red and White, as already reported in Sacchetto et al., 2009, Murgiano et al., 2012, Dorotea et al., 2015, respectively. All biopsies were collected under local anaesthesia during the course of routine clinical investigation. The national and institutional guidelines for the care and use of animals were followed and all procedures were approved by the local Institutional Animal Care and Use Committee. Muscle samples collected from healthy Chianina and Romagnola calves, were from animals killed at slaughterhouse and used as controls.

2.2. Immunohistochemistry

Muscle samples were frozen in cold isopentane. Transverse sections (8 μm) were cut in a cryostat and incubated with the mouse-monoclonal antibodies to SERCA1 (dilution 1:500, Biomol, Plymouth Meeting, PA, USA), recognizing an epitope between amino acid 506 and C-terminus of rabbit fast twitch SR Ca^{2+}-ATPase and with the mouse-monoclonal antibodies to SERCA2 (dilution 1:200, Biomol, Plymouth Meeting, PA, USA). Sections were then incubated with the secondary antibody conjugated with TRITC (tetramethylrhodamine isothiocyanate) (Dako, Milano, Italy). Serial sections from Romagnola pathological muscles were incubated with the same SERCA1 monoclonal

antibody described above or with the mouse-monoclonal antibodies specifically detecting sarcalumenin and the 53 kDa glycoprotein splice variant (dilution 1:200, Thermo Scientific). Sections were then incubated with the Alexa Flour Dyes-labelled anti mouse secondary antibody (Invitrogen). Confocal microscopy was performed using a Laica TCS-SP2 confocal laser scanning microscope.

2.3. Preparative procedures

A crude microsomal fraction enriched in content of SR membranes was isolated from PMT-affected Romagnola cattle muscle and by comparison from Romagnola healthy samples. Muscles were homogenized in 10 mM HEPES, pH 7.4, 20 mM KCl containing protease inhibitors 1 μg/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride as described (Sacchetto et al., 2009). The myofibrils were sedimented by centrifugation at $650 \times g$ for 10 min at $4\,°C$. The crude SR fraction was obtained from the previous supernatant by ultracentrifugation at $120,000 \times g$ for 90 min at $4\,°C$. Membrane fractions were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4 containing 1 μg/ml leupeptin and 100 μM phenylmethylsulfonyl fluoride and stored at -80 °C. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.4. Gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Slab gels were transferred onto nitrocellulose. The blots were probed with the following primary antibodies: mouse-monoclonal antibodies against SERCA1 (dilution 1:5000, Biomol, Plymouth Meeting, PA, USA), SERCA 2 (dilution 1:2000, Biomol, Plymouth Meeting, PA, USA), ryanodine receptor (RyR1, dilution 1:5000, Thermo Scientific), Sarcalumenin and glycoprotein53 splice variant (dilution 1:2000, Thermo Scientific). The transfer membrane was incubated with the secondary antibody alkaline phosphatase-conjugated (Sigma-Aldrich) and premixed BCIP/NBT solution (Sigma-Aldrich) was used to visualize the reaction.

2.5. Transmission electron microscopy analysis

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

3. Results and discussion

Cattle congenital PMT associated with *ATP2A1* gene mutations has been described in the Chianina, Romagnola breeds (Testoni et al., 2008; Murgiano et al., 2012) and in the Dutch improved Red and White cross-breed (Grünberg et al., 2010). The genetic defect underlying cattle PMT turned out to be heterogeneous, even though clinical signs and symptoms were homogeneous.

The effects of different mutations on the level of expression of SERCA1 protein have been extensively investigated by immunoblotting assay on microsomal fraction enriched of SR membranes (Sacchetto et al., 2009; Murgiano et al. 2012; Dorotea et al., 2015) as well as by immunofluorescence analysis of muscle cryosections. Here, we show transverse sections taken
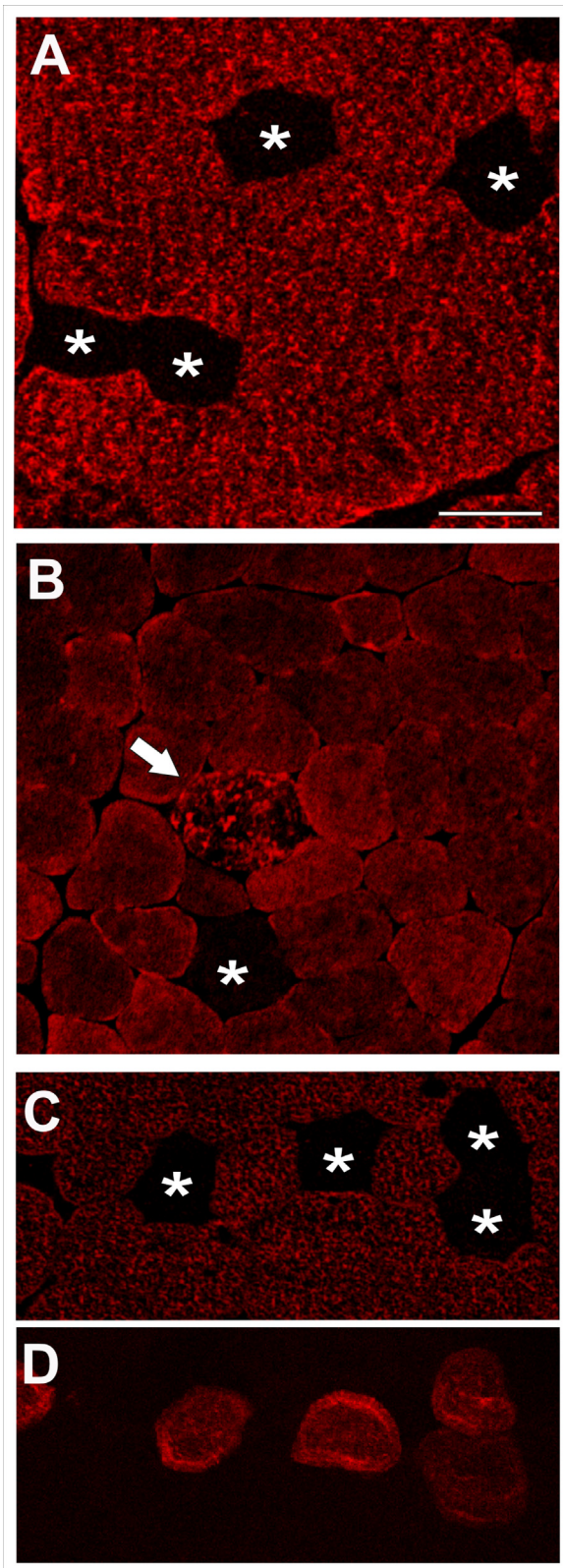
Fig. 1. Confocal microscopy of semimembranosus muscle cryosections. Muscle transverse sections from (a) healthy control and (b) PMT affected Romagnola calves were immunolabelled with monoclonal antibodies to SERCA1. Sections were then incubated with TRITC-conjugated anti-mouse secondary antibodies. Fibres unlabelled with this antibody, are indicated by asterisk. Arrow indicates enlarged degenerated fibre, filled with necrotic debris. The sections were processed in parallel and the images were taken at identical microscope and laser scanning settings. Serial cross-sections from Romagnola PMT-affected muscle were immunostained with antibodies to (c) SERCA1 or with monoclonal antibodies to (d) SERCA2. Fibres

from Romagnola pathological semimembranosus muscle, immunostained with a monoclonal antibody specific for the SERCA1 isoform (Fig. 1). Cattle semimembranosus is a representative fast-twitch muscle, composed mainly of fast-twitch fibres specifically expressing SERCA1 isoform, and only 8−10% slow-twitch fibres expressing the SERCA2 isoform. As already demonstrated in Chianina (Sacchetto et al., 2009) and Dutch improved Red and White (Dorotea et al., 2015), results showed that in Romagnola muscle sections as well, most fibres were immunolabelled with antibody for the SERCA1 isoform (Fig. 1b and c), with a small number of fibres unlabelled with this antibody. Fibres unlabelled with SERCA1 antibody were immunolabelled after incubation with antibody for SERCA2 (Fig. 1d). Although positive for SERCA1 antibody, the intensity of staining in fast-twitch fibres from PMT muscle was weak (Fig. 1b), when compared with control muscle (Fig. 1a). Moreover, rare fibres showed morphological alteration and staining aggregates, indicating degenerative phenomena (Fig. 1b).

The reduction in SERCA1 immunoreactivity in PMT-affected animals was confirmed by immunoblot analysis on microsomal fraction enriched in content of SR membranes with the same antibody (Fig. 2). By contrast, SR membranes from pathological and normal control muscles did not differ in the expression levels of junctional (ryanodine receptor) (Fig. 2) and non-junctional (sarcalumenina and its splice variant 53 kDa glycoprotein) (Fig. 2) SR protein markers, indicating that only SERCA1 is selectively affected. The loss of SERCA1 in pathological muscles was not compensated by the expression of SERCA2 isoform (Fig. 2).

By means of a heterologous HEK293 cellular model transfected with cDNA encoding R164H SERCA1 mutant, we recently used immunofluorescence analysis to study the cellular localization of the overexpressed SERCA1 pump. We reported that, as in PMT muscle samples, R164H SERCA1 was expressed at lower level than that of wild-type SERCA1, but it was correctly targeted to endoplasmic reticulum (ER) membranes of HEK293 cells, where it co-localized with calreticulin, a well know protein marker of ER membranes (Bianchini et al., 2014). Here, we investigated the association to SR membranes of SERCA1 mutants causing cattle PMT.

Jorgensen and co-workers (1979) showed that in rat muscles fibres stained with ATPase antibodies, this protein was uniformly distributed throughout SR membranes and formed a continuous polygonal staining pattern, consistent with the distribution of SR membrane network enveloping myofibrils. Transverse sections from all PMT-affected cattle breeds (Fig. 3b,c and d) and, by comparison, from bovine control healthy muscles (Fig. 3a), were immunolabelled with Ca^{2+}-ATPase SERCA1 antibodies. Fibres analysed by confocal microscopy at high magnification, revealed the same polygonal network already described in rat (Jorgensen et al., 1979) and other mammals, such as dog (Jorgensen and Jones, 1986) and rabbit (Sacchetto et al., 2002). An identical honeycomb-like staining pattern was observed after labelling transverse pathological muscle sections with antibodies to sarcalumenin/53 kDa glycoprotein splice variant proteins (Fig. 4a), located in the non-junctional SR where they co-localise with SERCA (Leberer et al., 1990). SERCA1 mutants, although expressed at lower level when compared with normal SERCA1, were correctly targeted to SR membranes.

The relevance of cattle PMT remains in its similarity to human Brody disease, a rare inherited disorder of skeletal muscle due to a SR Ca^{2+}-ATPase deficiency, resulting from a defect of *ATP2A1* gene

unlabelled with SERCA1 antibody, indicated by asterisks, are labelled with SERCA2 isoform, uniquely expressed in slow-twitch fibre in agreement with fibre-type composition of this muscle. Scale bar: 80 μm
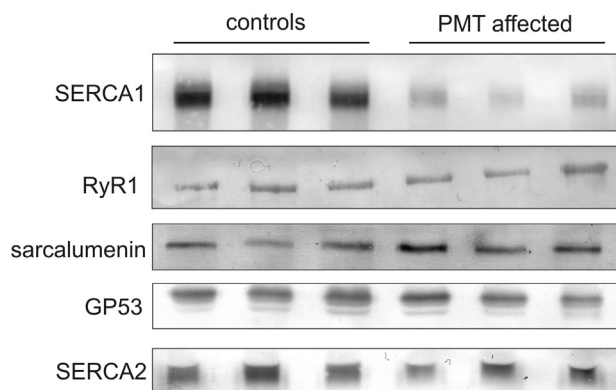
Fig. 2. Immunodetection of SERCA1 and of sarcoplasmic reticulum protein markers in controls and PMT-affected Romagnola muscles. Bovine semimembranosus muscles from healthy controls and PMT-affected Romagnola calves were homogenized and subfractionated to obtain a crude microsomal fraction enriched in SR membranes. An equal quantity of protein from microsomal fractions from controls and pathological samples was separated using 5–10% polyacrylamide linear gradient gel and blotted onto nitrocellulose. The blots were incubated with antibodies specific for SERCA1, calcium release channel/ryanodine receptor (RyR1), sarcalumenin, 53 kDa glycoprotein (GP53), and SERCA2, as indicated.
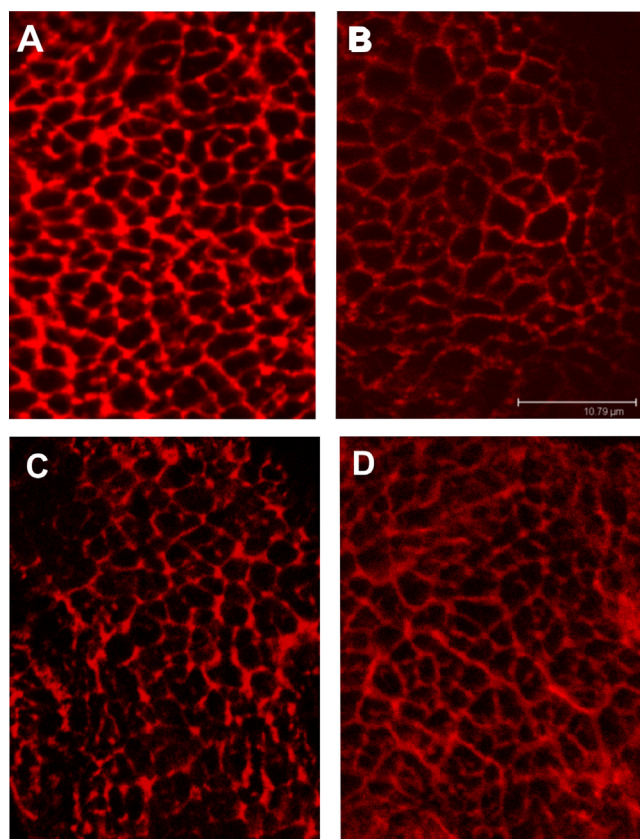

Fig. 3. Localization of SERCA1 wild-type and SERCA1 mutants in muscles from control and congenital PMT affected calves from different breeds. Semimembranosus muscle transverse cryosections from (a) Chianina control unaffected and PMT-affected animals from (b) Chianina (c) Romagnola and Dutch improved Red and White (d) breeds, carrying the R164H, G211V/G286V together with R164H, and R559 C SERCA1 mutations respectively, were immunolabelled with monoclonal antibodies to SERCA1. Sections were then incubated with TRITC-conjugated anti-mouse secondary antibodies. The sections were processed in parallel and the images were taken at identical microscope and laser scanning settings. Analysis of confocal fluorescence microscopy images were performed at high magnification. The honeycomb staining pattern observed in each of the sections from PMT specimens is very similar and equal to that of control healthy muscle. Scale bar: 10.79 μm.

(Odermatt et al., 1996). Brody disease is heterogeneous at both genetic and clinical picture levels (Voermans et al., 2012), although typical and common symptoms are exercise-induced muscle stiffness and difficulty in relaxation of skeletal muscle after repetitive contraction. The deficiency of SERCA1 protein at SR membranes is thought to be the cause of muscular stiffness observed in Brody patients. Impaired SERCA1 protein delays the removal of Ca^{2+} from cytoplasm back into the lumen of SR thereby prolonging the contractile phase. Nevertheless, the nature of SR Ca^{2+}-ATPase deficiency is still controversial. Some authors attributed the deficiency to a reduced protein expression (Karpati et al., 1986; Danon et al., 1988) in pathological muscles, while other groups showed that it was correlated to a reduction in Ca^{2+}-ATPase activity (Taylor et al. 1988; Benders et al., 1994).

It has been demonstrated (Karpati et al., 1986) that, in Brody muscle biopsies, triads as well as SR non-junctional component are normal in size and distribution. Nevertheless, a more recent study involving histological follow-up of a Brody patient has revealed progressive muscle alterations and morphologically abnormal nuclei (Mussini et al., 2015). As already demonstrated in our previous work (Sacchetto et al., 2009) using electron microscopy techniques, we confirm that, in PMT bovine muscle fibres, triads, although infrequent, were normal in size and distribution (Fig. 5a and b), but we failed to detect any nuclear abnormality.

Clinical symptoms, genetic and biochemical findings, clearly demonstrated that cattle congenital PMT is the true counterpart of human Brody disease. The pathogenetic mechanism underling cattle PMT has been definitively clarified in our recent work (Bianchini et al 2014). Using a combined experimental approach involving both HEK293 cells overexpressing R164H mutant SERCA1 and biopsies from Chianina cattle pathological muscles, we found that the ubiquitin-proteasome system (UPS) is involved in degradation of the SERCA1 mutant. Our results indicated that the mutation generates a protein most likely corrupted in proper folding, recovered by immunoprecipitation assay in a polyubiquitinated form, which was prematurely degraded by the UPS. We demonstrated that the treatment with proteasome inhibitors rescued the expression level of mutated SERCA1 at ER/SR membranes both in the HEK293 cell model and in muscle fibres from PMT-affected animals. Although mutated, R164H SERCA1 is functionally active. Therefore, by monitoring cell Ca^{2+}-fluxes with the Ca^{2+}-sensor protein aequorin in HEK293 cells or Ca^{2+}-ATPase activity in purified muscle microsomes, we demonstrated that the recovered R164H SERCA1 was able to re-establish resting cytosolic Ca^{2+} concentration.

At present, neither specific therapy nor mouse animal model for Brody disease exists. Brody patients are treated with muscle relaxant drugs preventing SR calcium release. However, due to liver toxicity, these drugs are unsuitable for long-term treatment. A SERCA1 knock-out mouse line was generated by Pan and co-authors (2003). Newborn SERCA1 null mice showed slow limb movements and contractures as in Brody patients. Nevertheless, diaphragm functions were severely impaired and neonatal mice died by respiratory failure within 2 h (Pan et al, 2003). The same Authors inferred that "in contrast to humans, SERCA1 is essential for survival in mice". This could explain why no SERCA1 knock-in mouse model has been generated up till now.

The identification of domestic animal genetic homologues of human pathologies is of increasing importance. Immunofluorescence analysis presented here shows that all *ATP2A1* mutations described until now in cattle breeds, although responsible for reduced protein expression, do not affect SERCA1 protein proper localization. These results, together with the evidence that no muscle fibres abnormalities are associated with PMT as well as Brody pathological conditions, prompted us to conceive possible
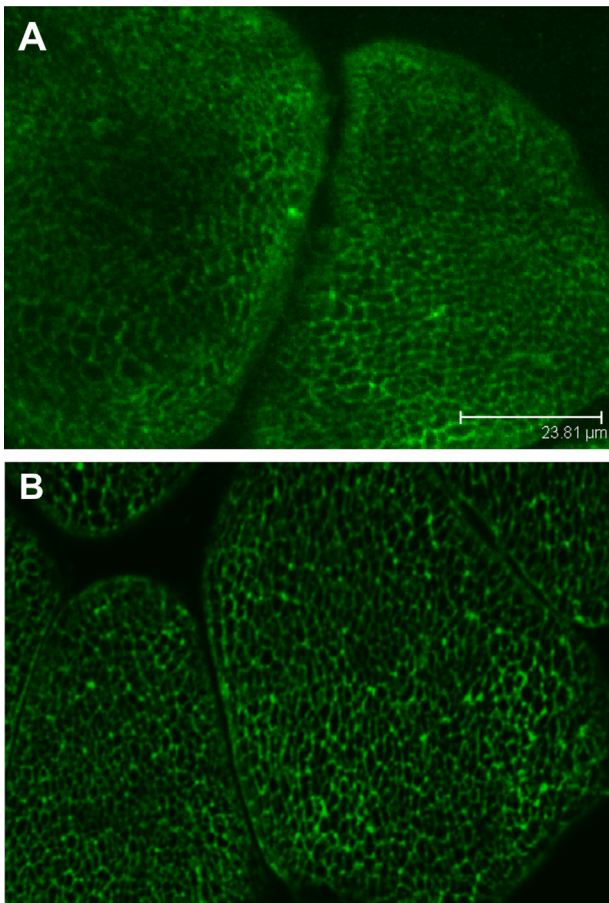
Fig. 4. Confocal microscopy of semimembranosus muscle cryosections from PMT affected Romagnola calves. Transverse cryosections from Romagnola PMT-affected calves were immunolabelled with monoclonal antibodies to sarcalumenin/glycoprotein53 (a) splice variant and with (b) monoclonal antibodies to SERCA1. Sections were then incubated with the Alexa Flour Dyes-labelled anti mouse secondary antibody. Analysis of confocal fluorescence microscopy images were performed at high magnification. SR Ca2 + -ATPase forms a 'honeycomb-like' pattern, when stained with anti-SERCA1 antibody, corresponding to the extensive sarcoplasmic reticulum membrane network. The same 'honeycomb-like' pattern can be observed when muscle sections from the same sample were stained with antibodies to sarcalumenin/53 kDa glycoprotein. Scale bar: 23.81 μm.

Fig. 5. Ultrastructural studies of bovine skeletal muscle fibres. Electron microscopy of longitudinal sections from (a) control unaffected and (b) PMT-affected Chianina bovine semimembranosus muscle biopsies. The structure of muscle fibre was normal and similar in both control and PMT affected muscles. Triads, although infrequent, are normal and are shown in high-power view in the inset. Scale bars: 500 nm.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

innovative pharmacological therapies based on the rescue of the mutated SERCA1 protein at SR membranes by acting specifically in the SERCA1 degradative pathway.

Nevertheless, in bovine PMT R164H, mutation does not abolish SERCA1 catalytic activity (Bianchini et al., 2014), while the R559C mutation, found in Dutch improved Red and White case, interferes with functional properties of the residual SERCA1 protein (Hua et al.2002; Clausen et al., 2003; Grünberg et al., 2010). The retention of functional properties of the mutated SERCA pump represents a prerequisite for the efficacy of potential innovative pharmacological therapies, since only fully functionally active SERCA1, when located at the SR membrane, could restore the efficient control of Ca^{2+} homeostasis and prevent the appearance of the pathological signs and symptoms.

In conclusion, cattle PMT is a faithful animal model of Brody disease which, although unconventional, permitted us to advance in knowledge of human pathology. This study reflects the enormous potential of an unconventional animal model to gain further insights into human medicine.
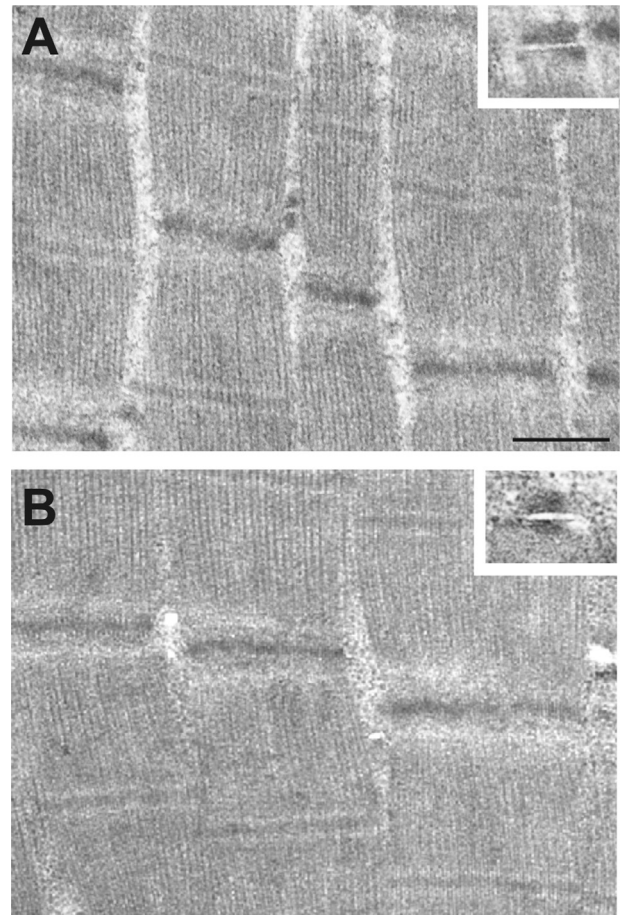
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