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Ultrastructure of diaphragm from dystrophic α-sarcoglycan-null mice✪

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α-Sarcoglycan is a 50 kDa single-pass transmembrane glycoprotein exclusively expressed in striated muscle that, together with β-, γ-, and δ-sarcoglycan, forms a sub-complex at the muscle fibre cell membrane. The sarcoglycans are components of the dystrophin-associated glycoprotein (DAG) complex which forms a mechanical link between the intracellular cytoskeleton and extracellular matrix. The DAG complex function is to protect the muscle membrane from the stress of contractile activity and as a structure for the docking of signalling proteins. Genetic defects of DAG components cause muscular dystrophies. A lack or defects of α-sarcoglycan causes the severe type 2D limb girdle muscular dystrophy. α-Sarcoglycan-null (Sgca-null) mice develop progressive muscular dystrophy similar to the human disorder. This animal model was used in the present work for an ultrastructural study of diaphragm muscle. Diaphragm from Sgca-null mouse presents a clear dystrophic phenotype, with necrosis, regeneration, fibre hypertrophy and splitting, excess of collagen and fatty infiltration. Some abnormalities were also observed, such as centrally located nuclei of abnormal shape, fibres containing inclusion bodies within the contractile structure, and fibres with electron-dense material dispersed over almost the entire cell. Additionally, unusual interstitial cells of uncertain identity were detected within muscle fibres. The abnormal ultrastructure of the diaphragm from Sgca-null mice is discussed.

Keywords: α-sarcoglycan, muscle ultrastructure, myopathy, sarcoglycan-deficient mouse, type 2D limb girdle muscular dystrophy

Dystrophin and dystrophin-associated glycoproteins (DAG) form a large complex at the sarcolemma of striated muscles (Blake *et al.*, 2002). The DAG complex forms a mechanical link between muscle fibre F-actin cytoskeleton and laminin-2 in the extracellular matrix (Ervasti & Campbell, 1993). The complex serves as a structure to protect muscle membrane from the stress of contractile activity, as well as to dock signalling proteins (Petrof *et al.*, 1993; Pasternak *et al.*, 1995; Henry & Campbell, 1996).

Sarcoglycans form a four-protein sub-complex of the DAG complex. They comprise α-, β-, γ, and δ-sarcoglycan, all single-pass transmembrane glycoproteins (Hack *et al.*, 2000). The precise role of the sarcoglycans within the DAG complex is not well understood but it has been proposed that they might be a recipient of signalling functions. α-Sarcoglycan has been in fact demonstrated to be an ecto-ATPase, i.e., an enzyme able to hydrolyze ATP and ADP (Betto *et al.*, 1999; Sandona *et al.*, 2004).

Genetic defects of DAG components may lead to muscular dystrophies: Duchenne/Becker dystrophy, associated to dystrophin protein defects, congenital dystrophy, due to laminin-2 absence, and sarcoglycanopathies, a subset of limb-girdle muscular dystrophies (LGMD) related to deficiency of the sarcoglycans (Straub & Campbell, 1997). Mutations in the α-sarcoglycan gene (*Sgca*), causing the lack or alterations of the protein, are responsible for type 2D LGMD, an autosomal-recessive severe human muscular disorder (Roberts *et al.*, 1994).

Animal models of human dystrophies are widely used to investigate pathogenesis, physiopathology, and treatment of these disorders (Durbeej & Campbell, 2002). Sgca-null mice have been generated recently, showing a milder pathological phenotype than the human disorder (Duclos *et al.*, 1998; Liu & Engvall, 1999). Histological analysis of hind-limb muscles showed dystrophic features that progress with age (Duclos *et al.*, 1998). The ultrastructure of those dystrophic muscles has not

✪Paper dedicated to the memory of Professor Witold Drabikowski and Professor Gabriela Sarzała-Drabikowska.

Abbreviations: DAG, dystrophin-associated glycoproteins; HE, hematoxylin-eosin; Sgca-null, α-sarcoglycan-null; LGMD, limb girdle muscular dystrophy.

been examined yet. In the present work, we performed an electron microscopy study of the diaphragm muscle from Sgca-null mice (Liu & Engvall, 1999), demonstrating unusual myopathic characteristics.

MATERIALS AND METHODS

Animals. The study was approved by the Ethics Committee of the Medical Faculty of the University of Padova. The experiments were carried out on 1.5 to 5 month-old Sgca-null male mice (Liu & Engvall, 1999) with the C57BL/6J strain (Charles River) as control. A minimum of four animals for ultrastructural and histochemical analyses was utilised. The animals were first weighed and then killed by cervical dislocation. Immediately after animal death, diaphragm muscle was excised, weighed, and attached to a plastic rod, as previously described in detail (Jakubiec-Puka, 1985), and immediately subjected to further procedures.

Histological analysis. For histological and histochemical studies, the muscles, attached to plastic rods and covered with tissue tek, were frozen in isopentane cooled in liquid nitrogen, and stored at –70°C. Transverse muscle sections (8 μm) were cut in a cryostat microtome (Leica CM 1850) set at –20°C. All procedures were performed as previously (Biral *et al.*, 2000). Histological and histochemical analysis were performed in serial muscle sections stained with hematoxylin-eosin and by the Gomori methods (Sarnat, 1983).

Electron microscopy. For ultrastructure study, muscle fixation, embedding and sample preparation procedures were applied as previously described in detail (Jakubiec-Puka, 1985). Ultrathin sections were prepared on an LKB Ultratome R III and then inspected using a JEM 1200 EX electron microscope. Sections were examined after contrasting with uranyl acetate and lead citrate, as previously described (Jakubiec-Puka, 1985).

Statistics. All data are expressed as mean ±S.E.M. Differences between control and Sgca-null mice were determined using unpaired Student's *t*-test. Statistical significance was established at $P < 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the analysis of body and diaphragm muscle mass of 2-, 3-, and 5-month old Sgca-null and control mice. Body mass of normal mice progressively increased with age, while that of Sgca-null animals increased from 2 to 3 months and was similar at 5 months (Table 1). The body mass of Sgca-null mice was always higher than that of age-matched controls, with the difference at 3 months being highly significant (Table 1). However, the difference between body mass of Sgca-null and control mice was progressively diminishing, being about 22% higher in 2- and 3-month old and only 5% in 5-month old Sgca-null mice with respect to controls. A similar trend was observed previously in knock-out mice not expressing α-sarcoglycan (Duclos *et al.*, 1998) and γ-sarcoglycan (Hack *et al.*, 1998), and in *mdx* mice (Pastoret & Sebille, 1995), even though the body mass of dystrophic animals was initially similar to that of wild-type controls and only later started to diminish. In contrast, the body mass of the dystrophic Bio 14.6 hamster, characterised by spontaneous absence of δ-sarcoglycan, was slightly lower with respect to wild-type controls but this difference did not change with age (Burbach *et al.*, 1987).

Diaphragm muscle from Sgca-null mice weighed significantly more than the age-matched controls (Table 1). The difference between the mean diaphragm muscle mass of Sgca-null mice compared to control muscles progressively diminished, still remaining significantly higher. In particular, diaphragm of 2 month-old Sgca-null mice was about 90% heavier than control, at 3 and 5 months of age the diaphragm was about 73% and 53% heavier, respectively, than control. The hypertrophy of diaphragm muscles is confirmed by the muscle-to-body mass ratio that is on average more than 50% larger in Sgca-null than in normal mouse. These data indicate that the hypertrophy of the diaphragm muscle of Sgca-null mice is elevated in young animals and tends to diminish with age. Hypertrophy of diaphragm is a frequent characteristic of all animal models for muscular dystrophies, as it has been reported in *mdx* (Lynch *et al.*, 1997), dystrophic hamster (Burbach *et al.*, 1987) and γ-sarcoglycan knock-out mice (Hack *et al.*, 1998).

Table 1. Body and diaphragm muscle weights of Sgca-null mice

Mass	2 months		3 months		5 months	
	control	Sgca-null	control	Sgca-null	control	Sgca-null
Body (g)	**21.6** ± 1.8 (3)	**26.3** ± 1.3 (4)	**28.8** ± 0.7 (4)	**35.4** ± 0.9 ** (4)	**30.1** ± 2.0 (3)	**31.7** ± 1.5 (3)
Diaphragm (mg)	**43.2** ± 9.1 (3)	**82.5** ± 17.1 (4)	**58.3** ± 5.7 (4)	**100.9** ± 6.6 ** (4)	**56.2** ± 2.7 (3)	**87.0** ± 8.7 * (3)
Muscle/Body	2.00	3.14	2.02	2.85	1.87	2.74

Data are means ± S.E.M.. Number of animals and diaphragm muscles analyzed are indicated in parenthesis. *, $P < 0.05$; **, $P < 0.005$, with respect to age-matched controls.

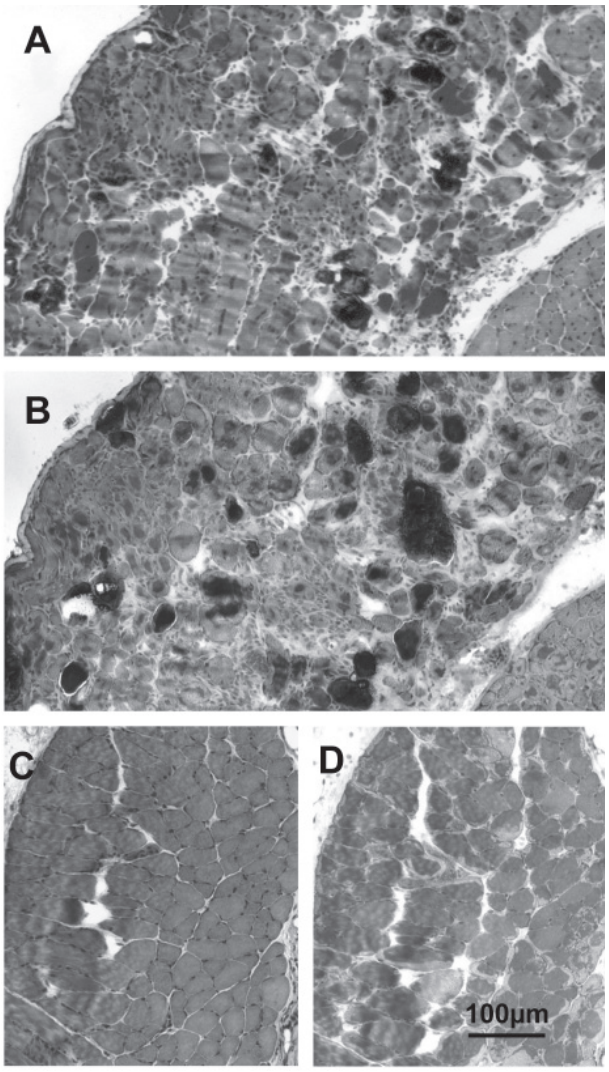

Figure 1. Light micrographs of transverse serial sections of diaphragm muscle from 5-month old Sgca-null (A, B) and control mice (C, D), stained with hematoxylin-eosin (A, C) and Gomori (B, D).

A. Sgca-null diaphragm showing muscle fibres of variable size and of non-uniform staining, large areas devoid of muscle fibres, centrally located nuclei, and some strongly basophilic fibres (dark staining). B. Sgca-null diaphragm showing several black areas, some of them corresponding to the basophilic muscle fibres of the HE stained specimens shown in section A. C and D. control diaphragm showing fibres of uniform size and staining intensity; all nuclei located at the periphery of muscle fibres.

Light and electron microscopy analysis of Sgca-null muscle revealed the presence of several abnormalities: fibre hypertrophy and splitting, areas of fibre necrosis and fatty infiltration, and fibre regeneration. These gross histological changes, which are typical for dystrophic muscle, were also previously described in diaphragm muscles of sarcoglycan-deficient animals (Burbach *et al*., 1987; Hack *et al*., 1998; Duclos *et al*., 1998; Durbeej & Campbell, 2002).

As shown by light microscopy analysis, the size of diaphragm muscle fibres of Sgca-null mice was variable: very large hypertrophying fibres were situated close to very small ones (myotubes, atrophic fibres or split fibre fragments) (Fig. 1A, B). In contrast, the size of diaphragm muscle fibres from control mice was relatively uniform (Fig. 1C, D). Several muscle fibres of the Sgca-null mice, especially those of larger size, were strongly basophilic when stained with hematoxilin-eosin (Fig. 1A). Some of them were within areas intensely stained by the Gomori method (Fig. 1B), suggesting the presence of non-specific phosphatase(s) activity.

Ultrastructural analysis of diaphragm muscle from Sgca-null mice reveals the presence of several abnormalities. In the interstitial areas between muscle fibres, we identified a discrete number of heterogeneous cells, large areas containing fields of irregularly arranged collagen fibrils (Fig. 2), split muscle fibre fragments and cell remnants. Some of the interstitial cells were identified as fibroblasts, leukocytes, lymphocytes, or mast cells, cells that are normally present also in control muscles. However, some of those cells showed features rather unique to normal muscle tissue. Particularly, we observed cells filled with lipofuscin-like material and cells containing very abundant rough endoplasmic reticulum (Fig. 2B). Moreover, some cells presented cell membrane processes and pseudopodia-like structures, forming connections with neighbouring cells.

The majority of fibres of the diaphragm muscle from Sgca-null mice contained centrally located nuclei, as shown by both light- and electron-microscopy (Figs. 1A and 3A, B), confirming previous observations in dystrophic animals (Hack *et al*., 1998; 2000; Duclos *et al*., 1998). However, the centrally located nuclei are characteristic of some congenital myopathies (De Angelis *et al*., 1991; Goebel, 2003). We often observed the centrally localised nuclei grouped or distributed in longitudinal rows along the muscle fibre (Fig. 3A, B). Interestingly, some of the nuclei showed an irregular shape and folding of nuclear membrane (Fig. 3A, B), even in the absence of evident signs of fibre damage or degeneration. On the other hand, shrinkage and fragmentation of nuclei and apoptotic-like nuclei were occasionally found within damaged or necrotic myocytes (not shown). As expected, muscle fibres from normal mice only rarely had centrally-located nuclei (Fig. 1C).

Ultrastructural analysis also shows the presence of several abnormalities. In some fibres numerous small vacuoles, resembling dilated T-tubules, and fat droplets usually close to mitochondria were observed. The vacuoles were situated among myofibrils or dispersed in the myoplasm. Several mitochondrial abnormalities were also observed, such as swelling, disruption or a giant size. Some of the damaged mitochondria contained myelin figures. Myelin figures were also present inside vacuoles but also dispersed in the extracellular space. These

pathological changes are not specific to Sgca-null muscles, as they are commonly observed in other muscular dystrophies.

The contractile structure of the diaphragm of Sgca-null mice looked like normal in most muscle fibres. However, occasionally, some fibres presented evident swelling and enlarged spaces between the myofibrils (Fig. 2A). Moreover, in some very small fibres, myofibrils were sparse, narrow and disordered. Additionally, in apparently well preserved fibres, some inclusion bodies-like structures were present within the contractile apparatus. These structures were tentatively identified as Z-line-related, being formed by loosely connected aggregates

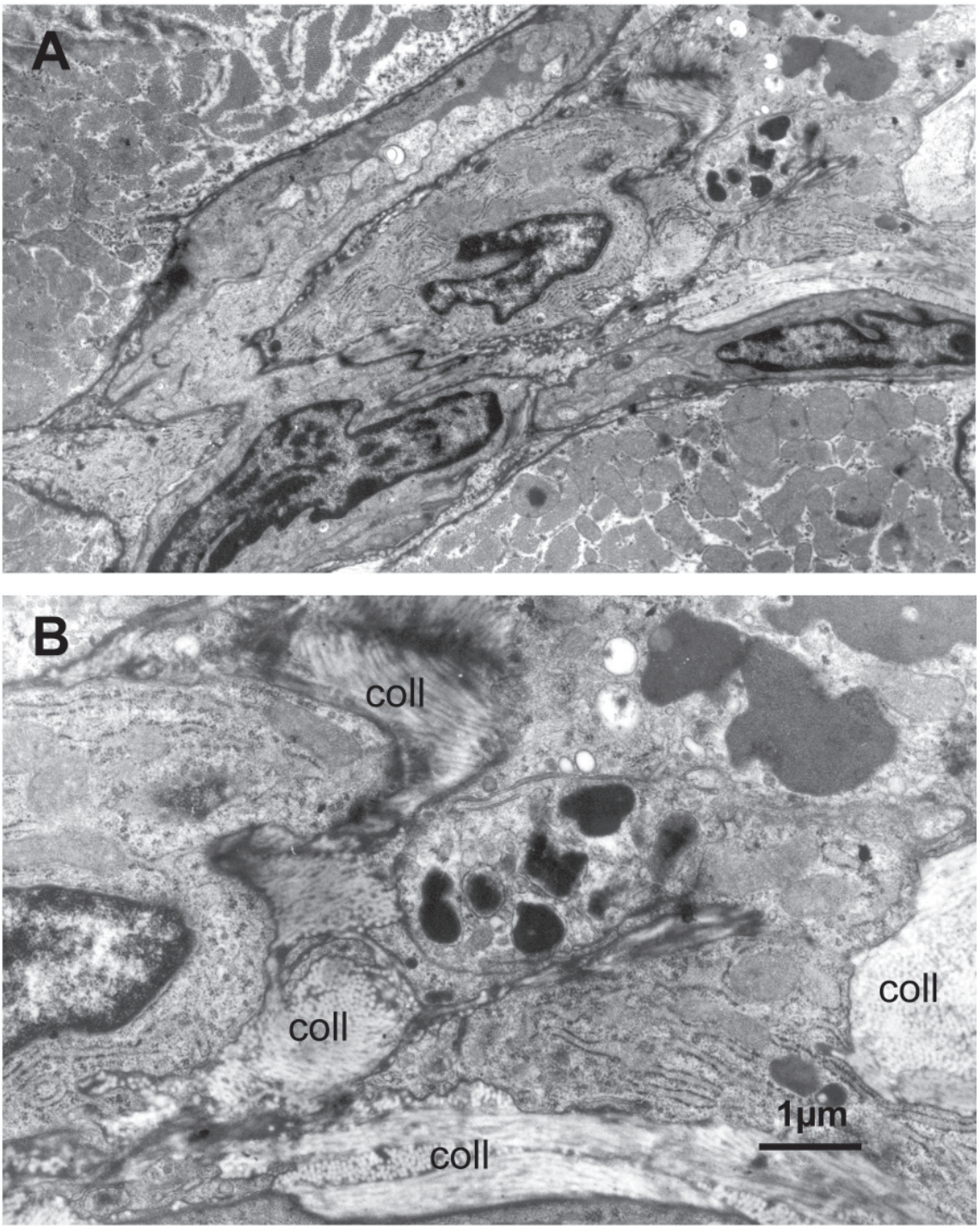

Figure 2. Ultrastructure of diaphragm of a Sgca-null 6-week-old mouse; transverse sections.

A. Among preserved muscle fibres a space is seen containing cells and collagen areas. Some swelling of muscle fibre in the left-hand upper corner; the cell in the right-hand upper corner contains some electron-dense remnants, the neighbouring one is like a mast cell. B. Higher magnification of the right-hand upper region of (A). Collagen fibrils (coll) irregularly arranged and differently oriented a cell rich in rough endoplasmic reticulum at the left-hand side.

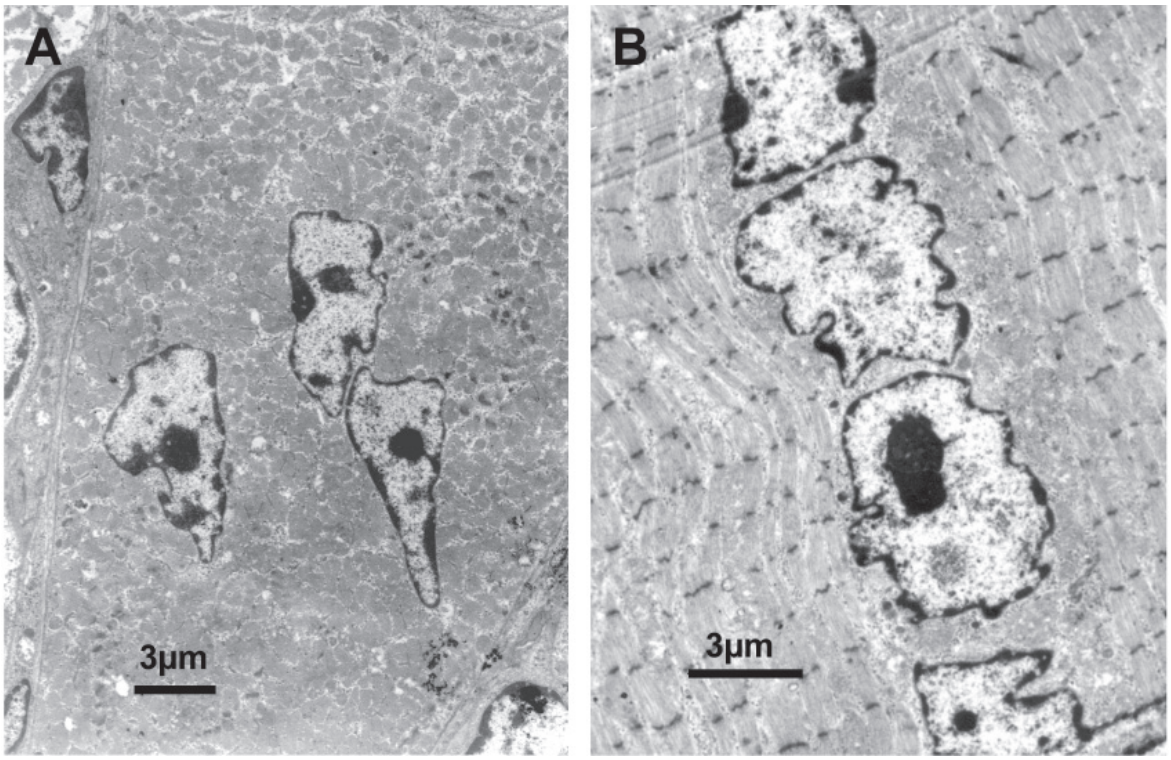

Figure 3. Ultrastructure of diaphragm of a Sgca-null 2-month-old mouse; transverse (A) and longitudinal (B) sections.

A and B. Centrally located nuclei situated in group (A) and row (B) within preserved contractile structure; the nuclear membrane folded, shape of nuclei irregular.

of fine filaments (Fig. 4). In fact, in some areas, these inclusions resembled the Z-line streaming (Fig. 4C, D), common in pathological muscles, observed also in muscles maintained in extended position (Jakubiec-Puka, 1985) or the Z-line forming in developing muscle (Fig. 4B). These inclusions, however, were usually (but not always) oriented in parallel with the myofibrils (Fig. 4C).

Inclusion bodies are a common feature of "protein aggregate myopathies". They are believed to be formed by the accumulation and aggregation of defective, abnormal proteins (Engel, 1999; Goebel, 2003). Inclusion bodies are, however, a very rare feature of muscular dystrophies. In fact, protein aggregates are not easily formed in muscular dystrophies characterised by selective "protein deficiencies" (Goebel, 2003). In contrast, surprisingly, filamentous aggregates/inclusions are evident within the contractile structure of apparently well preserved fibres of the Sgca-null mouse model of human type 2D LGMD. One can suppose that, analogously as it occurs in "aggregate myopathies", the pathological inclusions of Sgca-null diaphragm could be formed of proteins not directly affected by the genetic defect. These aggregates could be formed of one or more proteins whose expression is perturbed as a consequence of the lack of α-sarcoglycan. However, taking into account that α-sarcoglycan is a protein localised in the sarcolemma and that its function in the skeletal muscle is yet to be defined, it is difficult to envisage the nature of the aggregates. Since α-sarcoglycan was recently identified as an ecto-ATPase, it is possible that the aggregates found in dystrophic diaphragm could be related to the proposed function of α-sarcoglycan in controlling extracellular ATP signalling (Sandona *et al.*, 2004). However, the answer to this question requires more detailed studies of α-sarcoglycan and Sgca-null muscle.

An additional unusual observation made in some fibres of the Sgca-null diaphragm was the presence of electron-dense amorphous masses (Fig. 5). These masses were present either within selected fibre regions or encompassing nearly the whole fibre area, frequently associated with neighbouring electron-transparent areas. Those masses seem to surround muscle organelles, such as mitochondria, vacuoles or remnants of the contractile structures (Fig. 5B–E). However, close to the areas containing the dense masses there were also present normal-looking organelles: nuclei, mitochondria, sarcoplasmic reticulum, polysomes (Fig. 5). This indicates that these fibres are still alive, suggesting that they actively take part in the production of the electron-dense substance. Whether or not formation of the dense aggregates is a step toward muscle fibre death will be the matter of future investigations. An additional interesting issue is whether there exist some relationships between the formation of the amorphous masses, embedding the muscle fibre organelles, and the presence of Z-line-related structures and their

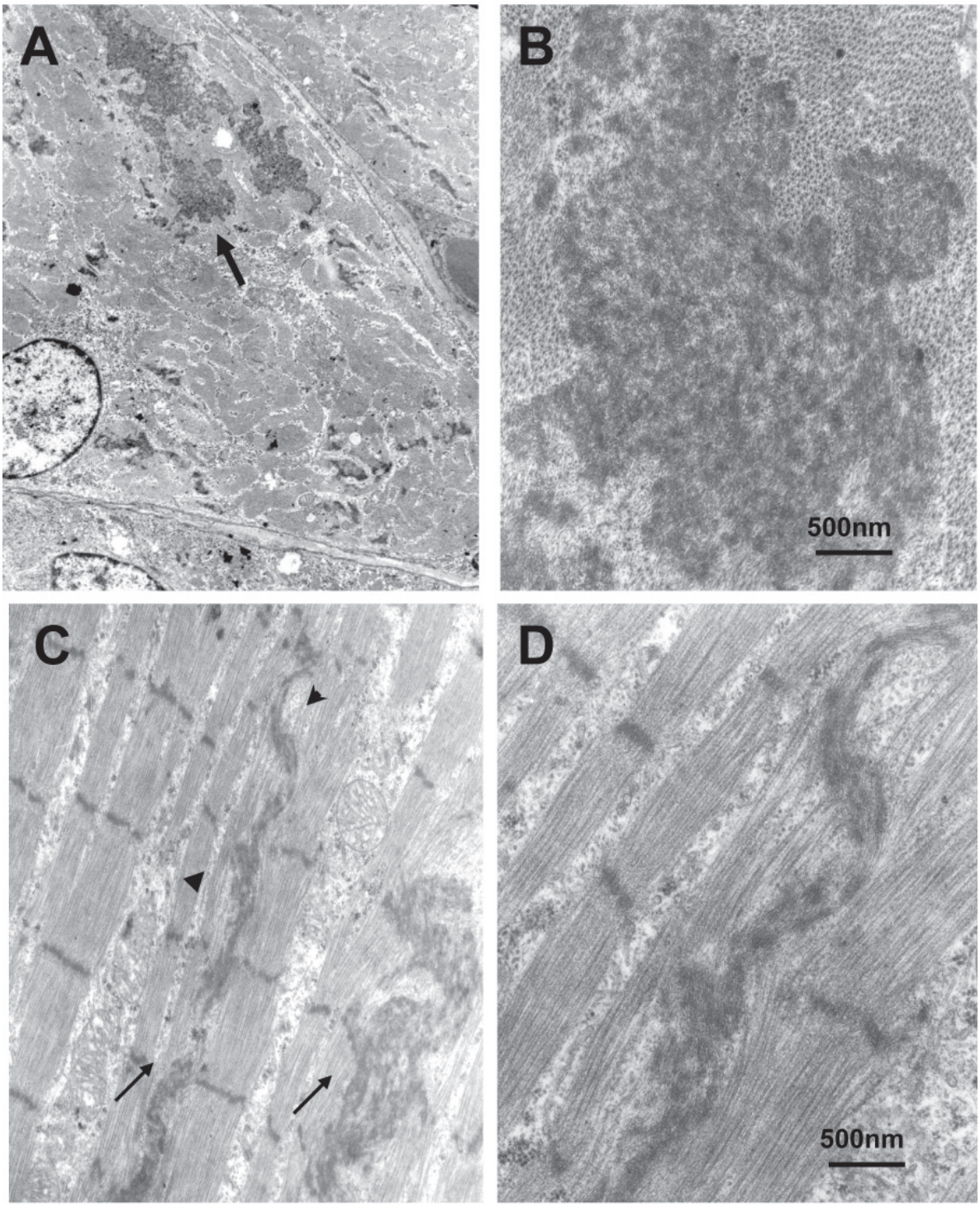

Figure 4. Ultrastructure of diaphragm of a Sgca-null 2-month-old mouse; transverse (A, B) and longitudinal (C, D) sections.

A and C. Inclusions within the contractile structure (arrows) of preserved muscle fibres. B and D. Higher magnification of (A, region shown with an arrow) and (C, region between arrowheads), respectively. The contractile structure preserved, but not regular; inclusions look like related to the Z-line (D) and resemble the Z-line forming in developing muscle (B).

relatively organised localization within the contractile apparatus. Last, but not least, a crucial issue is how to connect the observed unusual ultrastructural features of Sgca-null diaphragm with the lack of α-sarcoglycan in the sarcolemma. While this question will represent the matter of future work, the answer to it could provide new clues to revealing the pathogenetic mechanisms of muscular dystrophy.

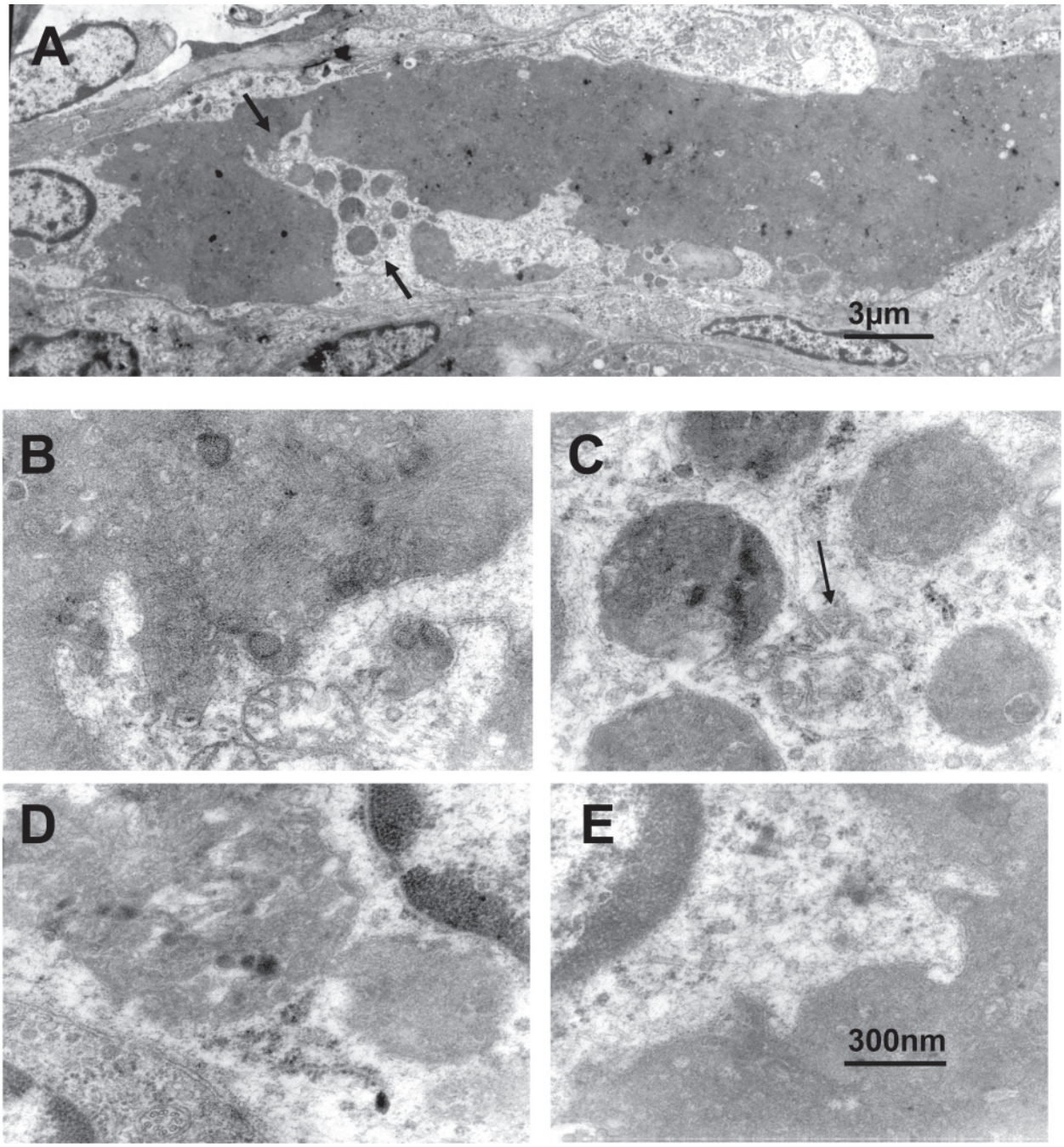

Figure 5. Ultrastructure of diaphragm of a Sgca-null 2-month-old mouse; transverse sections.

A. Muscle fibre filled within most of its area with electron-dense amorphous masses this fibre is surrounded with numerous cells. Regions indicated with arrows and the area surrounding the nucleus (the left-hand side) are shown in figures B–E. Traces of desorganized myofilaments and small vacuoles recognisable within the electron-dense masses (B). Mitochondria filled with electron-dense masses among them an electron-lucent mitochondrium with recognisable cristae (arrow) (C). Electron-dense masses present close to the preserved nucleus (D, E) containing dark bodies (D) and vacuoles (E). Polysomes, rough endoplasmic reticulum, vacuoles and some fine filaments are seen in areas close to the electron-dense masses (B–E).

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