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Cholesterol Depletion Uncouples β-dystroglycans from Discrete Sarcolemmal Domains, Reducing the Mechanical Activity of Skeletal Muscle

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Key Words

Cholesterol • Skeletal muscle • β-dystroglycan • Sarcolemma • Lipid raft

Abstract

Background/Aims: β-Dystroglycan (β-DG) is a transmembrane glycoprotein that links the intracellular cytoskeleton to the extracellular matrix and is crucial for the molecular pathway of lateral force transmission in muscle. We aimed to investigate the effect of decreasing sarcolemmal cholesterol on the distribution of β-DG, its interaction with dystrophin and the impact on the contraction efficiency of muscle. Methods: Isolated rat extensor digitorum longus muscles were incubated with methyl β-cyclodextrin (MβCD) to deplete cholesterol and with MβCD-cholesterol to restore cholesterol. Electric stimulation protocols were used to determine muscle force and fatigue. Detergent-resistant membranes (lipid rafts) were separated from isolated skeletal muscle sarcolemma. The distribution and interactions of β-DG, caveolin-3 and dystrophin were determined by an immunoreactivity analysis. Results: Cholesterol depletion in muscle results in a weakened force of contraction, which recovers after cholesterol restoration. The rate of fatigue is unaffected, but fatigue recovery is dependent upon cholesterol restoration. MβCD modifies the structures of lipid rafts obtained from MβCD-treated muscles by, displacing the membrane proteins β-DG and caveolin-3 f from the lipid raft, thus reducing the interaction of β-DG with dystrophin. Conclusion: Cholesterol depletion weakens the muscle contractile force by disturbing the sarcolemmal distribution of β-dystroglycan and its interaction with dystrophin, two key proteins in the alignment of lateral force transmission pathway.

Introduction

Dystroglycan (DG) is a 2-subunit protein derived from a single gene, DAG1, which encodes an 895-aminoacid precursor and is highly conserved in vertebrates. The precursor of DG is post-translationally cleaved between residues 653 and 654 to generate the

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α-DG and β-DG subunits [1]. α-DG is an extracellular peripheral membrane protein that associates with the muscle cell plasma membrane (sarcolemma) through an interaction with β-DG. Residues 550 and 565 of α-DG are non-covalently anchored to residues 691 and 719 of β-DG [2]. Importantly, the Cys^{669} and Cys^{713} residues of β-DG are redox regulated to control the interaction with the α-DG subunit [3]. DG is the central component of the dystrophin-glycoprotein complex (DGC) in muscle, which is linked to the transmission of lateral force during muscle activity [4, 5]. α-DG functions as a receptor for extracellular matrix proteins, such as laminin, thus ensuring contact between the basal membrane and the plasma membrane [6]. β-DG is a sarcolemmal transmembrane glycoprotein and contains a single 24-amino acid membrane-crossing helix. β-DG links the cytoskeleton to the extracellular matrix [1, 6, 7]. β-DG is aligned mainly with the Z-disk and exhibits a sarcomeric distribution along the myofibre [8]. The intracellular domain of β-DG interacts with the costameric proteins dystrophin and utrophin [9, 10]. Laterally, β-DG is also linked to the sarcoglycan protein complex (SGC) [11, 12]. The absence or mutation of any of the costameric proteins associated with the DG complex, such as dystrophin [13], dystroglycans [14], SGC components [10] and laminin [15], strongly perturbs the morphology of muscle fibres, resulting in weak and dystrophic muscle. The absence of dystrophin in skeletal muscle leads to a dramatic perturbation of the sarcolemmal location of both α- and β-DG [16].

Sarcoglycanopathies are a group of four autosomal recessive limb girdle muscular dystrophies (LGMDs) caused by mutations of the α, β, γ and δ sarcoglycan genes, resulting in the disruption of the sarcomeric arrangement of the SGC in the sarcolemma [17] and sarcoplasmic reticulum (SR) [18]. Some naturally occurring mutations in the DG gene have been associated with specific types of muscular dystrophy [19], but the absence of β-DG in skeletal muscle is embryonically lethal [20]. β-DG is the central component of the dystrophin-dystroglycan- laminin axis in striated muscle. During isotonic contraction, these proteins interact to transmit lateral force.

In adult skeletal muscle, the sarcolemma invaginates periodically, giving rise to the transverse tubule membrane network (TT-membrane). The TT-membrane openings to the sarcolemma are in close proximity to the Z-disk, and TT-membrane genesis is related to the formation of multiple caveolae, primarily in the I-band of the sarcomere [21, 22]. Caveolin-3, the muscle-specific form of caveolin, is also a major structural and regulatory integral membrane protein at the sarcolemma and in the TT-membrane. Caveolin-3-null mice exhibit abnormalities in the formation [23] and organisation of the T-tubule system [24] and have dilated and longitudinally oriented TT-membranes [25], thereby affecting sarcomeric fibre structure and fibre development [26]. Caveolin-3 is associated with different membrane regions of the cardiac sarcolemma and the TT-membrane that are related to hormone and drug signalling [27]. Signal transduction through cellular membranes can be regulated by the interaction of the cytoskeleton with caveolin-enriched membrane domains in striated muscle [28].

Caveolin-3 and β-DG are influenced by the lipid composition of the membrane. High cholesterol and sphingolipid concentrations in the plasma membrane are associated with discrete membrane domains [29] that are enriched with caveolins. The extraction of cholesterol from skeletal muscle fibre diminishes the interaction of caveolin-3 with dystrophin [30] and affects the contraction force of the muscle [31]. Because TT-membranes are enriched in cholesterol, cholesterol depletion may alter muscle mechanical activity by affecting the activity of the dihydropyridine receptor [31].

In the present study, we investigated the effects of decreasing the cholesterol concentration with methyl- β-Ciclodextryn (MβCD) on the mechanical properties of fast skeletal muscle. MβCD is a cyclic oligomer of glucopyranoside that does not incorporate into membranes, is not membrane permeable and selectively extracts membrane cholesterol by sequestering it in a central non-polar cavity [32]. From the isolated sarcolemma, we separated a ganglioside-enriched membrane with a detergent-resistant membrane domain (lipid raft) and analysed the distribution of β-DG. The depletion of sarcolemmal cholesterol with MβCD modified the distribution of β-DG in the membrane and the interaction of β-DG with dystrophin and caveolin-3. These effects were associated with a decrease in force development, which reversibly affected the mechanical properties of skeletal muscle. We propose that the disruption of discrete cholesterol-enriched domains in the sarcolemma affects the distribution of β-DG and, consequently, its interaction with dystrophin, resulting in a decreased force of contraction, which is immediately reversible upon the restoration of cholesterol content.

Materials and Methods

Animals

All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources of the United States as approved in Mexico by the Ethics Committee of the School of Medicine of the National Autonomous University of Mexico (UNAM) (NOM-062-ZOO1999).

Muscle preparation for mechanical studies

Male Wistar rats weighing 240 to 280 g were euthanised by cervical dislocation; the extensor digitorum longus (EDL) was then isolated at room temperature. The isolated muscle was placed in an acrylic chamber equipped with platinum electrodes along each side of the chamber wall that were in contact with the length of the muscle. Krebs solution (135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM dextrose, 1 mM Na_2PO_4 (dibasic), 15 mM $NaHCO_3$) was bubbled with 95% O_2 and 5% CO_2 to achieve a pH of 7.0. The EDL muscle was fastened from the distal tendon to forceps and from the proximal tendon to a force transducer. The platinum electrodes were connected in parallel to two stimulators (Grass SD9).

Maximal force at optimal sarcomeric length

Single electrical pulses of 0.6 msec were used to reach the maximal voltage for the maximal tension. The muscles were stretched to a length at which the twitch force was maximal to achieve the optimal sarcomeric length (2.4 μm). A tetanic stimulation of 75 Hz at 100 V for 1 sec was used, followed by 3 min of rest. This protocol was repeated several times with 3 min resting periods to ensure that at least 3 control tetanic forces were repeated. This force was considered the control maximal tetanic tension. At the end of the protocol, the muscle was allowed to rest for 10 min before the initiation of the fatigue protocol.

The EDL muscle length was 3.2 ± 0.6 cm, with a cross-sectional diameter of 6.2 ± 0.2 mm and a weight of 0.12 ± 0.01 g (s.d., n=9). The EDL muscle force (N) was determined from a calibration mass curve and the dimensions of the EDL muscle. The EDL dimensions were carefully observed to avoid a false impression on the calculated force between the experimental and control groups.

Tetanic fatigue protocol

Six tetanic stimulations of 75 Hz were given for 1 sec at 100 V with a one-minute rest between stimulations. After the six stimulations, a 5-min rest period permitted muscle force recovery. The muscle force in newtons (N) was determined from a calibration mass curve. The index of fatigability (fade) was calculated as the difference between the force recorded at the end of stimulation and the maximal force recorded for the particular tetanus.

Twitch fatigue protocol

A single twitch (100 V) stimulation train was used with 0.3 sec of rest between stimulations. Twenty minutes of stimulation was followed by 5 minutes of rest before the tension recovery experiment; the same stimulation protocol described above was used.

Cholesterol concentration in muscle fibre modified by MβCD

To study the effect of cholesterol removal on mechanical properties, the EDL muscle was incubated for 30 min with 15 mM MβCD and washed before the initiation of the fatigue protocol. To study the reversibility of the effect of cholesterol depletion on muscle activity, the EDL muscle was incubated with 15 mM MβCD and subsequently incubated with either 10 or 15 mM MβCD-cholesterol and washed before the initiation of the fatigue protocol. The normalised force was calculated from the maximal force produced in the same muscle during the initial tetanic response.

Quantification of cholesterol membrane content

To measure the cholesterol concentration, a cholesterol/cholesteryl ester quantisation kit (Biovision, CA, USA) was used according to the manufacturer's instructions.

Isolation of the sarcolemma

Eight rats were used for each sarcolemmal preparation. The sarcolemma was obtained from fast skeletal muscle (forelimbs, hind limbs and back muscles) by differential centrifugation and a discontinuous sucrose gradient with a modification of the method as previously described [33]. The cleaned muscle separated from the connective tissue was homogenized with a Polytron for 10 sec, followed by a 10-sec rest; this procedure was repeated two times. The membrane was isolated in the absence of a reducing agent in buffered medium (20 mM Tris-malate and 100 mM KCl, pH 7.0). The homogenate was centrifuged at 12,000 g for 20 min at 4°C. The supernatant was collected and filtered through four layers of gauze. Solid KCl was added to a final concentration of 0.6 M, and the sample was incubated for 1 hr with continuous stirring on ice. The homogenate was then centrifuged at 140,000 g for 40 min at 4°C. The precipitated fraction was suspended in a solution containing 20 mM Tris-malate and 100 mM KCl, pH 7.0, to eliminate excess KCl. The collected precipitated fraction was suspended in a solution containing 20 mM Tris-malate and 250 mM sucrose, pH 7.0, transferred to a sucrose gradient of 23%, 26%, 29% and 35% w/v and centrifuged at 75,000 g for 16 hr. The 23/26% interface showed the maximal signal for β-DG and low dihydropyridine receptor content as determined by immunoblotting. In addition, this fraction contained no SERCA1 enzymatic activity. Each fraction in the gradient was collected separately and suspended in a solution containing 20 mM Tris-malate and 100 mM KCl and centrifuged at 140,000 g for 40 min. The precipitated membranes from each fraction were collected and suspended in 500 μL of a solution containing 20 mM Tris-malate and 100 mM KCl and flash frozen with liquid nitrogen prior to storage at -20°C. The protein concentration was determined with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) with BSA as the standard.

Isolation of transverse tubule and sarcoplasmic reticulum membranes

The TT-membrane and SR membranes were obtained from fast skeletal muscles (forelimbs, hind limbs and back muscles). Three rats were used for each preparation. The isolation was performed by differential centrifugation with a discontinuous sucrose gradient as previously described [34]. The microsomal fraction was first placed in a sucrose gradient of 25 %, 27.5 %, and 35 % (w/v) and centrifuged at 75,000 g for 16 hr. The maximal signal for the dihydropyridine receptor was identified in the 25/27.5 % interface as determined by immunoblotting, indicating that it corresponded to TT-membranes. The isolated TT-membranes were incubated in a Ca^{2+}-loading solution in the presence of 5 mM potassium oxalate for further purification. An additional centrifugation in a discontinuous 25/45 % sucrose gradient was performed to remove the Ca^{2+} oxalate-loaded vesicles when required, as described [35]. The light SR was detected by the maximum ATPase activity stimulated by Ca^{2+}. The protein concentration was determined with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, USA) with BSA as the standard.

Isolation of lipid rafts from the sarcolemma

Lipid rafts were isolated as previously described [28, 36]. Briefly, aliquots of 0.1 mg/ml isolated sarcolemma were incubated for 30 min with a solution containing 1 % Triton X-100. After incubation, the membranes were gently mixed with an equal volume of 80 % sucrose (w/v) to give a final sucrose concentration of 40 % and placed at the bottom of an ultracentrifuge tube. The membranes were overlaid with 2.5 ml 30 % sucrose, followed by 1 ml 5 % sucrose and centrifugation at 170,000 g for 16 hr. Nine 0.5-ml fractions (excluding the pellet) were collected from the top of the gradient. The protein was suspended in an equal volume for each fraction, and the protein content was analysed by sodium-dodecyl-sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting under reducing conditions.

SDS-PAGE, western blot and dot blot

The protein samples were separated by SDS-PAGE (Pierce) on a 12 % polyacrylamide gel and transferred to nitrocellulose membranes (Millipore Corp). Immunoblotting was performed using the following antibodies: monoclonal anti-rat β-DG (1:500) and monoclonal anti-rat caveolin-3 (1:600) from Santa Cruz Biotechnology; anti-dihydropyridine receptor (IIID5) (1:800), anti-GLUT4 (1:10,000) and anti-actin (1:1000) from Abcam; and FITC-conjugated cholera toxin B (1:10,000) from Sigma. Affinity BioReagents were obtained from Sigma Chemical Co. After six 5 min rinses with a TBS-Tween/5 % (w/v), the samples were incubated with a secondary anti-mouse IgG antibody coupled to horseradish peroxidase from GE Healthcare (1:1250) for 1 hr at room temperature. All immunoblots were developed by enhanced chemiluminescence (Pierce).

Immunoprecipitation

The Triton X-100 fractions obtained from the sucrose gradient ultracentrifugation (200 µg of total protein) were subjected to immunoprecipitation overnight at 4°C with the anti-β-dystroglycan antibody pre-adsorbed onto protein A-Sepharose (Pharmacia, Uppsala, Sweden). The immunoprecipitates were treated and analysed by immunoblotting with the indicated antibodies. The nitrocellulose membrane was blocked with TBS-Tween in 0.05-5 % skim milk for 1 hr at room temperature prior to incubation with anti-β-dystroglycan, anti-dystrophin or anti-cav-3 antibodies overnight at 4°C. After extensive washing, the membrane was incubated with either anti-rabbit IgG or anti-mouse IgG antibodies coupled to horse radish peroxidase for 1 hr and washed again. Enhanced chemiluminescence (ECL and Super Signal ECL, Pierce) was detected with the Typhoon 9410 image station (Amersham) to obtain the net intensity values after background subtraction.

The standard deviation of the mean (± s.d.) was determined for all results.

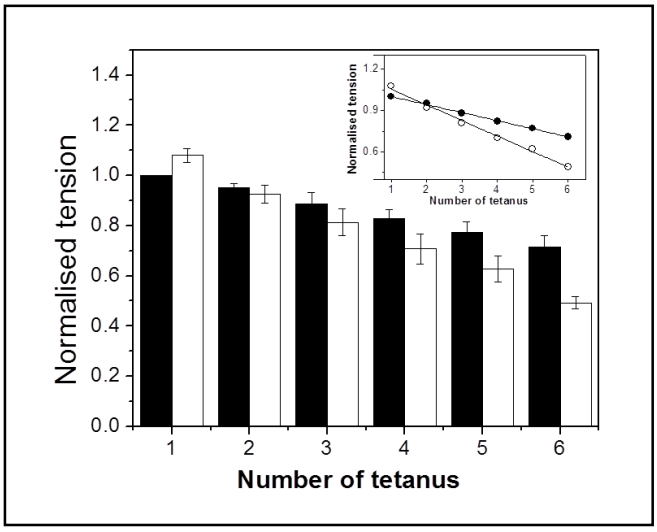

Fig. 1. The effect of cholesterol depletion on muscle fatigue during a tetanic contraction in control muscle (solid bars) and following MβCD incubation (open bars). The inset plot shows the linear representation of the rate of fatigue development in the control (●) and after incubation with MβCD (○). The results are the mean ± s.d. (n=5) for each condition.

Results

Effect of MβCD on the mechanical properties of the isolated extensor digitorum longus (EDL) muscle (tetanic stimulation protocol)

We used 15 mM MβCD to test the effect of a reduction of the cholesterol content on the whole muscle EDL contraction force. Figure 1 shows the effect of MβCD on the normalised force of six consecutive high frequency tetanic stimulations. The maximal tension of the control EDL was 1.16 N. We observed a 13%

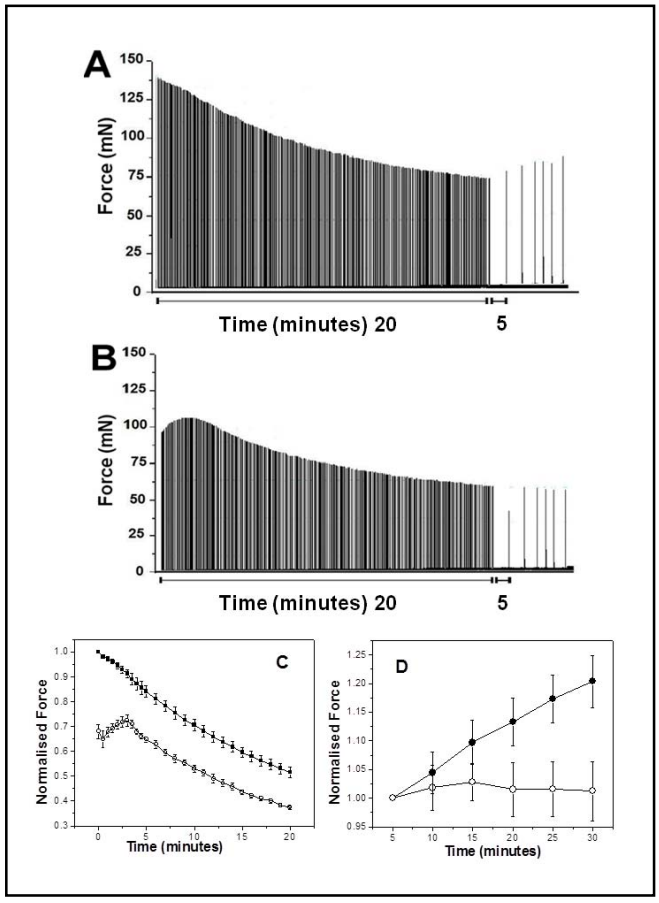

Fig. 2. The effect of cholesterol removal on fatigue development with a single twitch train stimulation protocol. A) Representative experiment in control EDL muscle and B) representative experiment in EDL muscle incubated with MβCD. C) Normalised tension of control EDL fatigue (●) and EDL incubated with MβCD (○). D) Normalised tension of fatigue recovery in control EDL (●) and EDL previously incubated with MβCD (○). The results are the means ± s.d. (n=5) for the control and (n=4) for the MβCD-treated muscle.

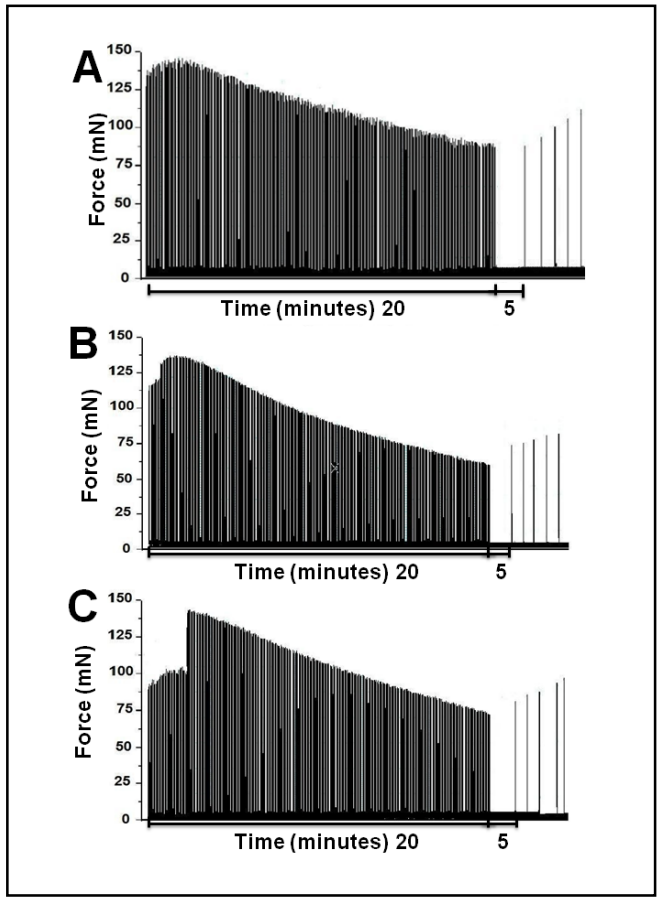

Fig. 3. The effect of cholesterol restoration on the mechanical properties of skeletal muscle EDL. A) Train of stimulation with single twitches of control muscle in the presence of 10 mM MβCD-CH. B) Train of stimulation with single twitches in the presence of 10 mM MβCD-CH following incubation with 10 mM MβCD for 30 minutes to deplete cholesterol. C) Train of stimulation with single twitches in the presence of 15 mM MβCD -CH following a previous incubation with 15 mM MβCD for 30 minutes to deplete cholesterol.

potentiation of the force in the presence of MβCD in the first tetanus with a force of 1.31 N. The potentiation disappears with the continued decay of the force in the following four tetanic contractions. The fatigue index was calculated as the ratio B/A, where B is the force recorded at the end of a tetanic contraction and A is the maximal force recorded at the beginning of the tetanus. The fatigue index was 0.97 in both the control and cholesterol-depleted EDL muscles. However, the force recorded after the sixth tetanus was 0.78 ± 0.05 N for the control EDL, which corresponds to a muscle force decay of 33% (s.d., n=5), and 0.50 ± 0.02 N for the cholesterol-depleted EDL muscle, which corresponds to a muscle force decay of 62 % (s.d., n=5), indicating that a 30 % lower force of contraction was elicited when the muscle was incubated with MβCD. The inset plot represents the force decay as a function of the six consecutive tetanic stimulations. The rate of fatigue (k) was k=0.5 for the control EDL muscle and k=0.26 for the MβCD-treated EDL muscle, i.e., fatigue occurred 48 % more quickly in the cholesterol-depleted muscle. MβCD affected muscle force and fatigue; however, it is unclear whether muscle fatigue is directly related to the depletion of cholesterol from plasma membranes. Thus, we used a single twitch fatigue protocol to further elucidate the effect of MβCD on EDL muscle fatigue.

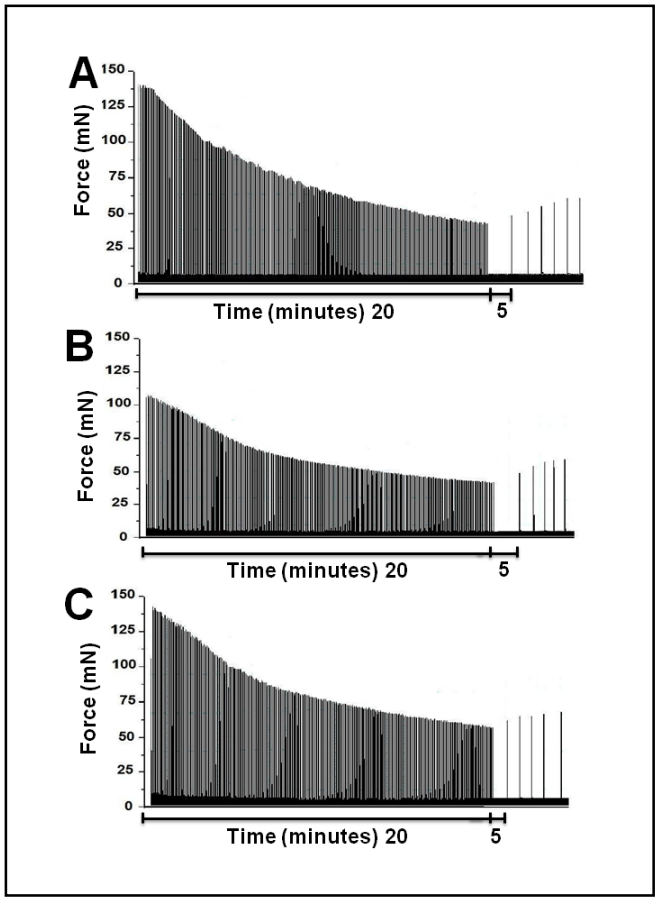

Fig. 4. The effect of caffeine on the mechanical properties of skeletal muscle EDL incubated with MβCD and MβCD-CH. A) Train of stimulation with single twitches in the presence of 10 mM caffeine. B) Train of stimulation with single twitches in EDL in the presence of 10 mM caffeine following incubation with 15 mM MβCD. C) Train of stimulation with single twitches in EDL in the presence of 10 mM caffeine following incubation with 15 mM MβCD-CH.

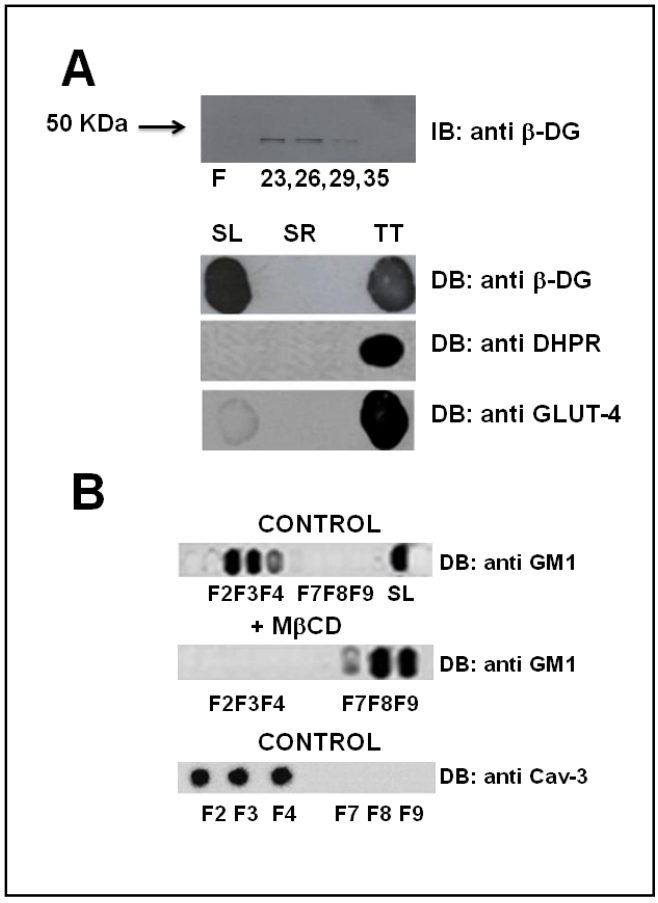

Fig. 5. Characterisation of isolated sarcolemma for lipid raft isolation. A) Western blot showing the enrichment of β-DG in the 23-26% fraction of the sucrose gradient and a dot blot of the isolated TT-membrane, sarcolemma and SR membranes with anti-dihydropyridine receptor, anti- β-DG and anti-GLUT-4 antibodies. B) The ganglioside GM1 concentration in the isolated lipid raft fraction was examined by a dot blot using cholera toxin B-HRP and anti-caveolin-3.

Effect of MβCD on EDL muscle fatigue

Single twitch train stimulation was used to observe the effect of MβCD on fatigue and recovery, as shown in Fig. 2. Figure 2A is a representative experiment with the control EDL, and Fig. 2B is a representative experiment with EDL previously incubated with MβCD. Figure 2C shows the average force of the normalised tension picked from different twitches along the time course of the stimulation train in the control and MβCD-treated EDL muscles. The contractile force at the first stimulus of the train was 0.44 ± 0.01N (s.d., n=5) for the control EDL muscle and 0.26 ± 0.01 N (s.d., n=4) for the cholesterol-depleted EDL muscle. The muscle tension in the control EDL decreased by 55 % ± 2 of the initial force (s.d., n=5) after a 20 min stimulation with a linear tendency. EDL depleted of cholesterol exhibited a 41 % ± 3 (s.d., n=4) reduction in tension in the first single twitch with respect to the control. Although the force in the cholesterol-depleted EDL muscles decreased drastically at the first stimulations, the rates of fatigue in the control and cholesterol-depleted muscles were the same. However, there was a consistent period, corresponding approximately to the first 2 min of the train stimulation, in which the muscle force in the cholesterol-depleted EDL increased by 10 %. Figure 2D shows the normalised force of recovery after fatigue, which measures the muscle fibre integrity. The control EDL exhibited up to a 70 % recovery of the force of contraction after several stimu-

lation-resting events, whereas EDL incubated with MβCD did not recover after the fatigue protocol, suggesting muscle cell damage. Therefore, we investigated the effect of cholesterol restoration with MβCD-cholesterol (MβCD-CH) on the contractile force and muscle fatigue recovery in whole EDL muscle.

Reversible effects of MβCD on EDL muscle mechanical properties following cholesterol restoration

To examine whether the restoration of cholesterol to the membrane in the whole EDL muscle produced a reversible effect on muscle mechanical properties, we incubated EDL muscle in MβCD-cholesterol (MβCD-CH) for 30 min without a previous cholesterol depletion treatment (control). In Fig. 3A, we observed a force increase during the first two minutes of the stimulation train in the control muscle, but the overall force did not change, suggesting that MβCD and not cholesterol depletion is responsible for the 10 % increase in muscle force. The EDL muscle was incubated for 30 min with 10 and 15 mM MβCD (Fig. 3B, C, respectively) to observe a dose-dependent cholesterol depletion effect. After cholesterol depletion, the EDL muscle was incubated with 10 mM MβCD-CH and exhibited an immediate and complete restoration of the muscle force and, interestingly, a full force recovery after fatigue. The decreased force in the presence of MβCD strongly suggests that the cholesterol was removed from the surface membrane and that the force decay was dependent on the MβCD concentration. However, the increased tension during the first stimulations in the presence of either MβCD or MβCD-CH indicated a putative intracellular calcium concentration increase that was not directly related to the cholesterol depletion. To investigate if the SR was the source of the extra calcium released into the myofilament space, we used caffeine to stimulate the release of calcium from the SR in the presence of MβCD.

Effect of caffeine on EDL muscle contractile force and fatigue in MβCD-treated muscle

The increased tension observed during the stimulation train to reach fatigue in EDL muscle previously treated with MβCD (Fig. 2B) or with MβCD-CH (Fig. 3) suggested that the remaining calcium in the SR was released. Figure 4A shows the effect of 10 mM caffeine on the mechanical activity of control EDL muscle and on EDL muscle that was previously incubated with 15 mM MβCD (Fig. 4B) or 15 mM MβCD -CH (Fig. 4C). The

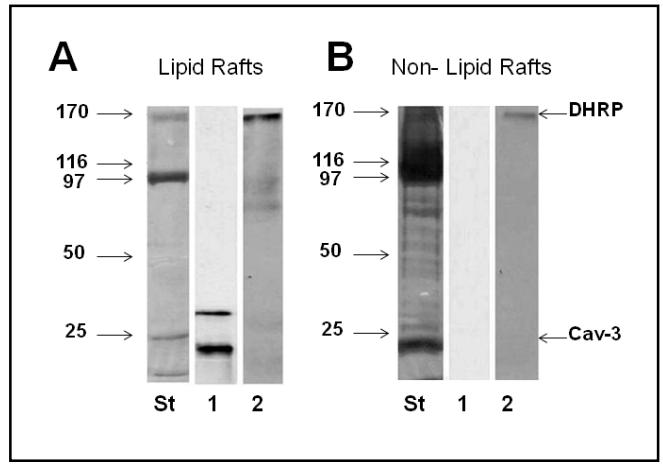

Fig. 6. Lipid rafts isolated from the TT-membrane. Western blot of the isolated transverse tubule membranes with 1) anti-caveolin-3 antibody and 2) anti-dihydropyridine receptor antibody. A) Lipid raft and B) non-lipid raft fractions.

force potentiation effect of MβCD and MβCD -CH during the first 2 min of the fatigue protocol (shown in Fig. 2B and Fig. 3) disappeared in the presence of caffeine. Nevertheless, the decrease in contractile force after cholesterol depletion was consistently observed, as was the effect of MβCD -CH on the recovery of contractile force after muscle fatigue.

The cholesterol depleted from the membrane in striated muscle can come from two possible sources of surface cholesterol: the sarcolemma and the TT-membranes. We further investigated the effect of MβCD on the isolated sarcolemma from fast skeletal muscle, specifically focusing on the distribution of β-DG in the sarcolemma as the main component of lateral force transmission.

The sarcolemma is organised into discrete lipid raft domains.

Lipid rafts isolated from highly purified sarcolemma

The β-DG-enriched membrane fraction observed by immunoblotting was obtained from the sucrose gradient interface (23 and 26 %) as described in the Materials and Methods. These two interfaces were collected as the sarcolemma fractions (Fig. 5A). Figure 5A shows the results of the dot blot analysis for the major molecular markers, β-DG, dihydropyridine receptor and glucose transporter-4 (GLUT-4), which are used to characterize the sarcolemma (SL) and TT-membrane (TT). The isolated light SR membrane (SR) was used as a negative control. The highest level of immunoreactivity in the isolated sarcolemma was observed with the β-DG marker. A lower level of immunoreactivity was observed for

GLUT-4, and no immunoreactivity was observed for the dihydropyridine receptor, confirming the successful isolation of the sarcolemmal fraction. The TT-membrane immunoreactivity was highest for the dihydropyridine receptor, GLUT-4 and β-DG. The immunoreactivity of the SR membrane was negative for all three markers.

The sarcolemma was incubated with Triton X-100 to isolate the lipid raft fraction as described in the Materials and Methods. The distribution of the membrane along the gradient was collected in nine fractions. Figure 5B shows that fractions 2-4 had the highest content of the ganglioside GM1 and caveolin-3 and fractions 7-9 lacked immunoreactivity for both GM1 and caveolin-3. Of the total sarcolemma protein content, 90 % was found in the heavy fraction, which consisted of the detergent-soluble membrane, while β-DG was detected mainly in the light fraction, i.e., the detergent-resistant membranes, also known as lipid rafts. The depletion of cholesterol with MβCD, which affected the mechanical properties of the muscle, also affected the distribution of β-DG in the membrane.

The incubation of the sarcolemma in 15 mM MβCD decreased the cholesterol content by 12%, from 0.220 to 0.190 ± 0.002 μmol/mg protein (s.d., n=4). The cholesterol/phospholipid (CH/PL) ratio was 0.2 in the sarcolemma, 0.9 in the TT-membranes and 0.02 in the light SR membranes isolated from fast skeletal muscle. The high concentration of cholesterol in the TT-membranes raised the question of whether the TT-membrane was organised in discrete domains such as lipid rafts, which are enriched in cholesterol and sphingolipids and would, therefore, be vulnerable to the depletion of cholesterol with 15 mM MβCD.

Lipid rafts isolated from TT-membranes

The TT-membrane has high concentrations of cholesterol and sphingolipids. The fractionation of the TT-membrane with Triton X-100 yielded a light fraction (detergent-resistant membrane), which corresponded to 90 % of the total lipid membrane, and a heavy fraction (detergent-soluble membrane), which contained 95 % of the total TT-membrane proteins. Figure 6A shows that caveolin-3 and the dihydropyridine receptor were mainly present in the detergent-resistant membranes as detected by immunoblot analysis. Figure 6B shows thatcaveolin-3 was absent in the detergent-soluble membranes. The dihydropyridine receptor was also present in the detergent-soluble fraction but in a lower amount. Because β-DG is known to be mainly located in the sarcolemma and partitions in the lipid raft fraction, we investigated the effect of sarcolemmal cholesterol depletion on the sarcolemmal distribution of β-DG.

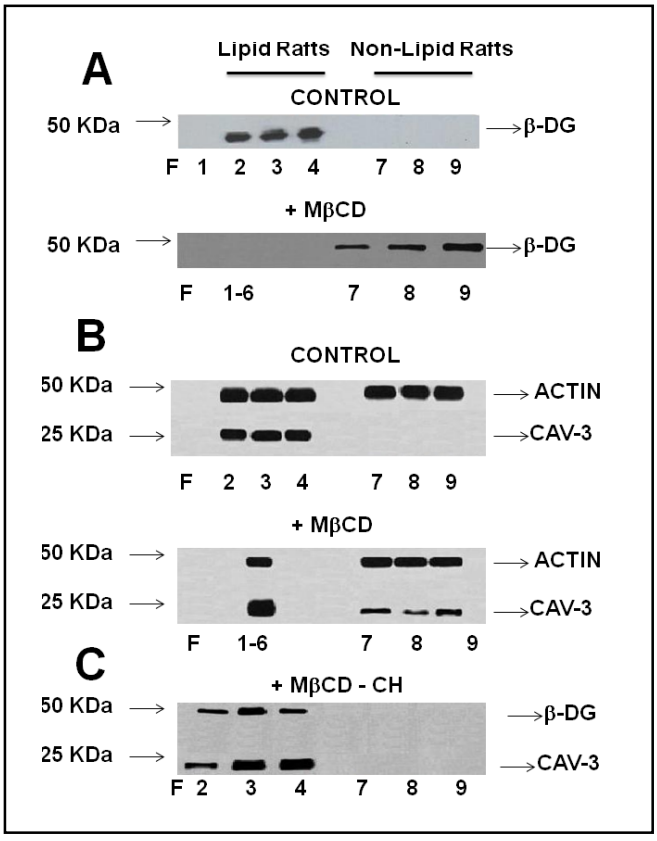

Fig. 7. The effect of MβCD on the distribution of β-DG and caveolin-3 in the membrane. A) Western blot with anti- β-DG antibodies of the lipid raft and non-lipid raft fractions isolated from the sarcolemma in the absence of MβCD (top) and in the presence (bottom) of 15 mM MβCD. B) Western blot with anti-caveolin-3 of the lipid raft and non-lipid raft fractions isolated from the sarcolemma in the absence of MβCD (top) and in the presence of 15 mM MβCD (bottom). C) Western blot with anti-β-DG and anti-caveolin-3 of the lipid raft and non-raft fractions previously incubated with 15mM MβCD and thereafter incubated with 15 mM MβCD-CH.

β-Dystroglycan and caveolin-3 sarcolemmal distribution changes with MβCD

Figure 7 shows an immunoblot of the lipid raft and non-lipid raft sarcolemmal fractions in the absence and presence of MβCD. Figure 7A shows the preferential distribution of β-DG in lipid rafts and the decrease in the amount of β-DG in the lipid raft in the presence of MβCD. Figure 7B shows the preferential distribution of caveolin-3 in lipid rafts. In the presence of MβCD, only a fraction of the caveolin-3 was released from the lipid raft. Figure 7C shows that the restoration of the cholesterol content to the sarcolemma by MβCD-CH resulted in the restoration of β-DG and caveolin-3 to the lipid raft. The re-

lease of β-DG from the lipid raft in cholesterol-depleted membranes indicated the misallocation of a key transmembrane costameric protein essential for the dystrophin-DG-laminin alignment, which represents a molecular mechanism implicated in lateral force transmission. To examine if the β-DG/dystrophin interaction was affected in the cholesterol-depleted lipid raft, we performed an immunoprecipitation analysis.

Immunoprecipitation of β-dystroglycan with caveolin-3 and dystrophin in lipid rafts isolated from sarcolemma depleted of cholesterol by MβCD

Figure 8 shows the co-immunoprecipitation of β-DG with caveolin-3 and with dystrophin. In lipid rafts, where caveolin-3 and β-DG are concentrated, there was no observable interaction between caveolin-3 and β-DG. In the non-lipid raft fraction, the small amount of β-DG present also did not interact with caveolin-3. The depletion of cholesterol in either the lipid raft fraction or in the non-lipid raft fraction induced the β-DG/caveolin-3 interaction. The β-DG/caveolin-3 interaction was 1.3-fold higher in the non-lipid raft membrane fraction than in the lipid raft fraction in cholesterol-depleted membranes. For the co-immunoprecipitation of β-DG with dystrophin, we first used a Typhoon 9410 image station to analyse the blots and obtained no signal (empty row in Fig. 8).The anti-dystrophin blot could only be developed following a long incubation with a high sensitivity ECL substrate that can detect proteins at the femtogram level. The co-immunoprecipitation of β-DG with dystrophin revealed an interaction in the lipid raft fraction that decreased by 37 % in the cholesterol-depleted sarcolemma. β-DG also interacted with dystrophin in the non-lipid raft fraction; this interaction decreased by 42 % when the sarcolemma was depleted of cholesterol.

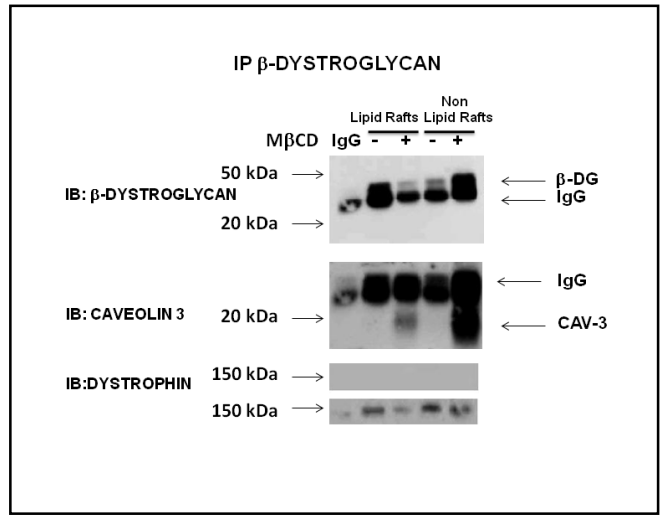

Fig. 8. The co-immunoprecipitation of β-DG with dystrophin and caveolin-3. The pooled Triton X-100-insoluble (lipid raft) and -soluble (non-lipid raft) fractions from sucrose gradient ultracentrifugation that had been treated (+) or not treated (-) with MβCD were immunoprecipitated with an anti- β-DG antibody and analysed using10% SDS-PAGE. The proteins were transferred to nitrocellulose membranes and immunoblotted with an anti- β-DG antibody (upper lane). The same blot was probed with anti-caveolin-3 (middle panel) or anti-dystrophin (lower panel) antibodies. The blots were visualised using enhanced chemiluminescence with a Typhoon 9410 image station, but the anti-dystrophin signal could not be detected. The anti-dystrophin blot was only visible following a long incubation with a high-sensitivity ECL substrate, which provides a femtogram level of protein detection.

Discussion

The results of this study demonstrate that MβCD, which reduced the sarcolemmal cholesterol concentration, delocalized β-DG from sarcolemmal lipid rafts enriched in the ganglioside GM1 and caveolin-3, promoted the interaction of β-DG with caveolin-3 in the non-lipid raft fraction of the membrane and interfered with the β-DG/dystrophin interaction. A decrease in the absolute force of contraction was observed when the mechanical properties of cholesterol-depleted fast skeletal muscle were measured. Although the rate of fatigue was faster in the tetanic fatigue stimulation protocol in cholesterol-depleted muscle, the rate of fatigue with a single twitch stimulation train protocol was not affected, suggesting that a different mechanism was responsible for muscle fatigue in cholesterol-depleted muscle. The recovery of force after fatigue in cholesterol-depleted muscle was prevented until cholesterol was restored to the membrane. MβCD and MβCD-CH increased the force during the first two minutes of the stimulation train, and the addition of caffeine during the electric stimulation of the muscle eliminated the increase in the force, indicating an indirect effect of MβCD on SR calcium regulation.

The increased membrane cholesterol concentration in the isolated surface membranes of different cells has been shown to inactivate a variety of membrane proteins, such as ion transporters [37-39], channels [40, 41] and receptors [42-44]. The TT-membranes in rat skeletal muscle contain a significant amount of cholesterol with a cholesterol/phospholipid (CH/PL) ratio of 0.9, simi-

lar to the ratios of 0.5 to 0.9 that have been observed by other research groups in amphibians [45], poultry [46] and rabbits [34]. The depletion of cholesterol from whole muscle by MβCD reduces the current amplitudes of L-type Ca^{2+}channels, which are located mainly in the TT-membrane, and does not affect the current amplitudes of T-type Ca^{2+}channels, which are located mainly in the sarcolemma of embryonic muscle [31]. Because the depletion of cholesterol by MβCD in isolated muscle cells does not have an effect on the propagation of the action potential, it has been suggested that cholesterol depletion specifically targets the dihydropyridine receptor [31]. A direct effect of cholesterol on the activities of other membrane proteins, such as the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) [47] and the plasma membrane Ca^{2+}-ATPase (PMCA) in cardiac sarcolemma [38], has been demonstrated. In striated muscle, the surface membranes have two well-defined pools of cholesterol: the sarcolemmal lipid rafts, which represent only a fraction of the sarcolemma, and the TT-membranes, which are enriched in cholesterol. Therefore, the depletion of cholesterol from whole muscle by 15 mM MβCD may have a different effect on membrane cholesterol efflux. In several cell types, the depletion of membrane cholesterol by MβCD occurs through at least two different kinetic stages of cellular cholesterol efflux: a fast accessible pool with a half-time of 19-23 s and a slow accessible pool with a half-time of 15-30 min [48].

Mechanical properties of fast skeletal muscle partially depleted of cholesterol

The maximal force of the control EDL during a tetanic contraction was 150 kN/m^2. Although the contractile force of EDL in the rat model has been reported to be 250 kN/m^2 [49], the animals used in this study had twofold lower body weight, muscle weight and muscle cross sectional area than the animals in the cited study. The difference in force between the control and cholesterol-depleted muscles depended only on the cholesterol or MβCD concentration and not on the muscle dimension. The force produced by tetanus or single twitch contractions continued with normal excitation contraction coupling after the depletion of cholesterol, but the overall force of the EDL in cholesterol-depleted muscle was reduced. The functional integrity of the isolated EDL muscle following the experimental procedure was confirmed by the full recovery of the force after fatigue. Therefore, the weakened force in the MβCD-treated muscles indicates the critical role of membrane cholesterol concentration in the maintenance of muscle force. Because MβCD is not membrane-permeable, the effect observed on the muscle force is related to the reduction of the cholesterol concentration in surface membranes. During tetanic contraction, the SR is depleted of calcium and does not have time to recover due to the reduced activity of the SR calcium pump (SERCA1), which leads to muscle fatigue [50]. Based on the results shown in Fig. 1, we propose that the enhanced tension of the first tetanic contraction of cholesterol-depleted muscle could be a result of dihydropyridine receptor activation, as was previously suggested [31]. However, consecutive tetanic contractions in muscle partially depleted of cholesterol resulted in force decay with a faster rate of fatigue. Tetanic contraction is directly related to calcium release from the SR, and the rate of relaxation is directly related to the activity of SERCA1 because high frequency stimulation does not allow calcium to be pumped back to the SR quickly enough to recover for the next stimulus. To investigate the effect of cholesterol depletion on the development of fatigue without the complication of potential effects on SERCA1, the EDL was stimulated with a train of single twitches to reach fatigue.

Effect of MβCD on muscle fatigue by a train of single twitch stimulations

In a muscle partially depleted of cholesterol, the increased activity of the dihydropyridine receptor has been suggested to shorten the time for excitation contraction coupling due to its fast interaction with the RyR [31]. However, EDL muscle incubated with MβCD exhibited an immediate loss of force during the complete time course of the single twitch train and maintained the same rate of fatigue as that observed in control muscle, which is the opposite of what has been observed with the muscle tetanic fatigue protocol and suggests that the depletion of cholesterol had no effect on excitation-contraction coupling but, rather, an effect on force transmission. Because cholesterol depletion affects membrane proteins, we propose that the effect of cholesterol depletion on the overall reduction of the muscle force is due to a failure in the transmission of lateral force as a result of the delocalisation of β-DG from cholesterol-enriched membrane domains and the reduced β-DG/dystrophin interaction.

The restoration of cholesterol to muscle fibres by MβCD -CH led to a full recovery of the contractile force, which indicated that the deficit in force transmission in the fibre could be reverted after a fatigue and demonstrated that the partial depletion of cholesterol

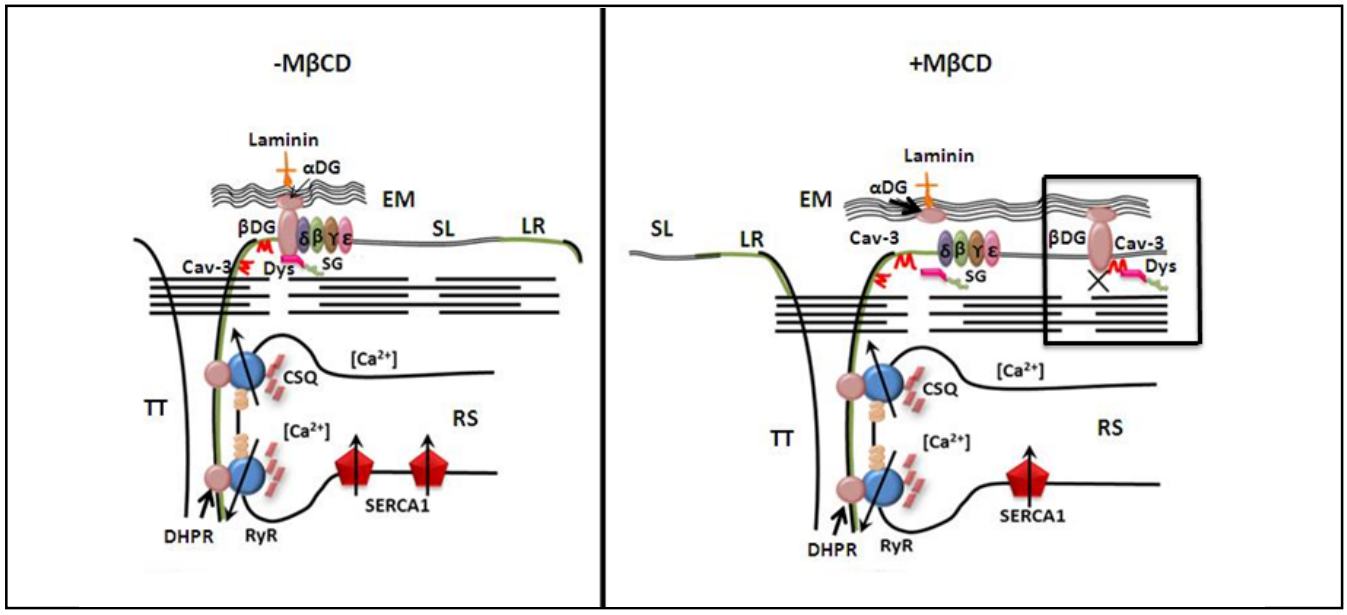

Fig. 9. A model of the effect of membrane cholesterol depletion on the sarcolemmal distribution of β-DG. The sarcolemmal lipid rafts close to the clear opening of the TT-membrane are enriched in cholesterol, GM1(ganglioside M1), Cav-3 (caveolin-3) and β-DG (dystroglycan). The schematic diagram illustrates the diminished contact between β-DG and Dys (dystrophin) in the presence of MβCD (methyl β-cyclodextrin); the β-DG/Dys interaction is essential for lateral force transmission. SERCA1 (sarcoplasmic reticulum calcium ATPase) and RyR (ryanodine receptor (SR Ca^{2+} channel)) function normally. SL, sarcolemma; SR, sarcoplasmic reticulum; EM, extracellular matrix; LR, lipid raft; Dys, dystrophin; SG,sarcoglycan; CSQ, calsequestrin.

did not damage the muscle cells but, rather, uncoupled the transmission of force. A comparable fatigue protocol in EDL muscle obtained from mice with muscular dystrophy resulted in irreversible damage [5, 18]. In cholesterol-depleted muscle, the observed immediate mechanical recovery of the EDL muscle after cholesterol restoration suggested that the interaction of membrane proteins with the sub-sarcolemmal cytoskeleton is strongly dependent on the sarcolemmal cholesterol concentration.

The increased force observed in all MβCD- or MβCD-CH treated muscles at the beginning of the stimulation was eliminated by the addition of caffeine, suggesting an indirect effect of MβCD on SR calcium regulation that was not due to cholesterol depletion. SERCA1 from the isolated light SR of skeletal muscle previously incubated with either MβCD or MβCD-CH exhibited increased ATPase hydrolytic activity. However, MβCD added directly to the isolated SR had no effect on SERCA1 activity (data not shown). The mechanism of this indirect effect is under investigation in our laboratory. Although the ability of MβCD to increase force was eliminated by caffeine, the decreased force due to the depletion of cholesterol was consistent throughout the stimulation train.

Localisation of β-DG in lipid rafts and its co-immunoprecipitation with caveolin-3 and dystrophin in cholesterol-depleted membranes

β-DG is mainly located in the Z-line in close proximity to the sarcolemmal clear TT-membrane openings. The presence of β-DG in the isolated TT-membranes indicates that a fraction of the TT-membrane co-purifies with the sarcolemma, but this does not necessarily signify sarcolemmal contamination by TT-membranes as neither GLUT-4 nor the dihydropyridine receptor could be observed in significant amounts in the isolated sarcolemmal fraction. The presence of caveolin-3 is an indication of caveolae formation [51] and is required for the formation of the TT-membrane system [26]. The direct interaction of β-DG with caveolin-3 has been described in NIH 3T3 fibroblast cells. A caveolin-3 WW-like motif domain competes with the WW-like motif domain of dystrophin for the PPxY-domain of β-DG and has been suggested as a mechanism involved in the failure of protein-protein interactions in Duchenne muscular dystrophy [52]. However, in a previous investigation using isolated membranes from skeletal muscle, affinity chromatography demonstrated that no direct interaction exists between β-DG and caveolin-3 [9]. We showed that,

in skeletal muscle, caveolin-3 is embedded only in sarcolemmal lipid rafts and on TT-membranes and has no interaction with β-DG until the cholesterol is removed. The interaction of β-DG and caveolin-3 outside of the lipid rafts suggests that one or both of the proteins assumed a different conformation. The difference observed in the β-DG/caveolin-3 interactions in non-muscle cells [53] and sarcolemma isolated from fully differentiated skeletal muscle [9] may be related to the complexity of the surface membrane system in a fully differentiated muscle fibre. The interaction of caveolin-3 with β-DG and dystrophin in cholesterol-depleted membranes may serve as an anchor to prevent further membrane damage.

The most significant finding in the present study was the observation that the delocalisation of β-DG from the lipid raft is dependent on the cholesterol content and affects the interaction with dystrophin. We propose that this alteration in the β-DG/dystrophin interaction provides the mechanism by which the contractile force is decreased in muscle treated with MβCD and then restored after the restoration of cholesterol to the membrane (Fig. 9). The removal of cholesterol has been previously shown to result in the delocalization of lipid raft proteins in several other cell systems [29, 36, 42]. The co-immunoprecipitation of β-DG with dystrophin in the lipid raft fraction demonstrates that the removal of cholesterol decreases the interaction with dystrophin. Nevertheless, β-DG and dystrophin interact either inside or outside of the lipid raft. In skeletal muscle, the existence of a non-costameric β-DG fraction present in neuromuscular junctions [54] and in intercostameric regions [54] has been suggested. However, there have been no studies on isolated membranes from neuromuscular junctions that corroborate this assumption; the small fraction of β-DG present in the non-lipid raft fractions of the sarcolemma may come from non-costameric membrane-associated domains. The key function of β-DG is to transmit lateral force to the extracellular matrix through its interaction with dystrophin in the alignment of the Z-disk proteins. The disruption of this interaction has been proposed as the causative mechanism in many muscular dystrophy diseases, especially in Duchenne muscular dystrophy, in which dystrophin is absent.

Lipid rafts are receiving increasing attention as cellular structures that contribute to the pathogenesis of several functional processes. Nevertheless, little is known about the roles that the composition of lipid rafts may have in the development of an important series of diseases related to muscle function. The presence of proteins in lipid rafts depends on their lipid and protein content. Current membrane models incorporate clusters of proteins and lipids and consider a dynamic remodelling according to the inclusion or exclusion of components. Lipid rafts participate actively in both signal transduction and cellular adaptation to many challenges through changes in their composition and environment. However, the mechanisms underlying such changes and the compartmentalization of the different molecules remain poorly understood. Although extensively studied, we still do not understand the mechanisms by which β-dystroglycans, which are members of the dystrophin-associated protein complex along with g-actin, the sarcoglycans, and the syntrophin-dystrobrevin scaffold for signalling proteins, may be modulated by several lipid raft components, including cholesterol. The present study presents evidence that a decreased concentration of cholesterol in lipid rafts significantly affects muscle force transmission by delocalizing β-dystroglycan from specific membrane domains.

Because our findings demonstrate that fluctuations in cholesterol concentrations in specific membrane domains of the muscle cell drastically affect muscle function, cholesterol-lowering pharmacologic agents used for the treatment of hypercholesterolemia may require review based on case reports that suggest that treatment with these drugs may lead to neuromuscular degeneration [55].

Acknowledgements

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