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Drastic reduction in the luminal Ca^{2+}-binding proteins calsequestrin and sarcalumenin in dystrophin-deficient cardiac muscle

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Abstract

Luminal Ca^{2+}-binding proteins play a central role in mediating between Ca^{2+}-uptake and Ca^{2+}-release during the excitation–contraction–relaxation cycle in muscle fibres. In the most commonly inherited neuromuscular disorder, Duchenne muscular dystrophy (DMD), the reduced expression of key Ca^{2+}-binding proteins causes abnormal Ca^{2+}-buffering in the sarcoplasmic reticulum (SR) of skeletal muscle. The heart is also affected in dystrophinopathies, as manifested by the pathological replacement of cardiac fibres by connective and fatty tissue. We therefore investigated whether similar changes occur in the abundance of luminal Ca^{2+}-regulatory elements in dystrophin-deficient cardiac fibres. Two-dimensional immunoblotting of total cardiac extracts was employed to unequivocally determine potential changes in the expression levels of SR components. Interestingly, the expression of the histidine-rich Ca^{2+}-binding protein was increased in the dystrophic heart. In contrast, the major Ca^{2+}-reservoir protein of the terminal cisternae, calsequestrin (CSQ), and the Ca^{2+}-shuttle and ion-binding protein of the longitudinal tubules, sarcalumenin, were drastically reduced in cardiac mdx fibres. This result agrees with the recently reported decrease in the Ca^{2+}-release channel and Ca^{2+}-ATPase in the mdx heart. Abnormal Ca^{2+}-handling appears to play a major role in the molecular pathogenesis of the cardiac involvement in X-linked muscular dystrophy.

Keywords: Muscular dystrophy; Cardiomyopathy; mdx; Dystrophin; Calsequestrin; Sarcalumenin

1. Introduction

The most common gender-specific genetic disease in humans is represented by X-linked Duchenne muscular dystrophy (DMD), a neuromuscular disorder affecting skeletal muscles, the heart and the nervous system to a varying degree [1–3]. Primary genetic abnormalities in the DMD gene lead to a deficiency in the membrane cytoskeletal protein dystrophin [4] and a significant reduction in a surface-associated glycoprotein complex [5]. Since the dystrophin–glycoprotein complex stabilises the fibre periphery in normal muscle providing a trans-sarcolemmal linkage between the actin membrane cytoskeleton and the extracellular matrix [6], loss of this complex impairs the integrity of the sarcolemma [7]. The calcium hypothesis of muscular dystrophy assumes that a weakened plasma membrane structure renders the muscle surface more susceptible to microscopical membrane rupturing [8]. During the natural processes of surface membrane resealing, Ca^{2+}-leak channels are introduced into the dystrophic sarcolemma causing increased cytosolic Ca^{2+}-levels in dystrophin-deficient muscle fibres [9]. A recent report by Mallouk et al. [10] suggests that the Ca^{2+}-elevations occur not throughout the sarcoplasm but are located near the sarcolemma. The rise in the cytosolic Ca^{2+}-concentration contributes to a pathophysiological cycle of enhanced protease activity and subsequent Ca^{2+}-leak channel activation [9].

Since changes in Ca^{2+}-homeostasis may trigger severe muscle fibre damage in neuromuscular disorders, the fate of the Ca^{2+}-regulatory apparatus has been the subject of numerous investigations into the pathology of muscular dystrophy [8–13]. Comparative equilibrium dialysis revealed that the overall Ca^{2+}-buffering capacity of the

Abbreviations: CSQ, calsequestrin; DMD, Duchenne muscular dystrophy; Dp427, full-length dystrophin isoform of 427 kDa; HRCBP, histidine-rich calcium binding protein; mdx, X-linked muscular dystrophy mouse; SAR, sarcalumenin; SR, sarcoplasmic reticulum

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dystrophic sarcoplasmic reticulum (SR) is reduced by approximately 20% [12]. It is believed that the drastic reduction in the calsequestrin (CSQ)-like proteins CLP-150, CLP-170 and CLP-220 [12] represents impaired CSQ clustering [14]. This may lead to changes in the co-operative kinetics within CSQ clusters causing reduced ion-binding that may contribute to a decreased luminal SR Ca^{2+}-buffering capacity. In addition, the expression of the Ca^{2+}-shuttle protein sarcalumenin (SAR) is approximately 70% lower in mdx fibres as compared to normal skeletal muscle [13]. Pathophysiologically, the reduced ion-binding capacity of the luminal SR probably amplifies elevated free cytosolic Ca^{2+}-levels and thereby accelerates the dystrophic protein degradation process in skeletal muscle fibres.

Besides severe skeletal muscle degeneration [15], the clinical progression of X-linked muscular dystrophy also encompasses impaired cardiac function in the majority of patients [16]. Since cardiomyopathy is the cause of death in a significant proportion of DMD cases [2], it is of central importance to understand the pathobiochemical pathways leading to dystrophic abnormalities in the heart [17]. A widely employed animal model of DMD is represented by the mdx mouse [18], which is missing the full-length Dp427 isoform of dystrophin due to a spontaneous point mutation in exon 23 [19]. Although the mdx heart is not a perfect replica of the cardiac involvement in the human disease [2], its dystrophic phenotype is clearly of clinical relevance [20]. The mdx heart exhibits markedly altered contractile properties [21] and displays necrotic changes and inflammation [22], and mdx mice show an abnormal electrocardiogram [23]. Thus, the dystrophic phenotype of the mdx heart represents a suitable DMD model to investigate potential changes in Ca^{2+}-handling proteins due to a deficiency in dystrophin. The 2D immunoblotting analysis of total cardiac mdx extracts presented here demonstrates a reduced expression of two major luminal Ca^{2+}-binding proteins of the SR, CSQ and SAR. This suggests that the calcium hypothesis of muscular dystrophy can, at least partially, be extrapolated from skeletal muscle to cardiac fibres.

2. Materials and methods

2.1. Materials

Protease inhibitors were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Chemiluminescence substrates were obtained from Perbio Science UK (Tattenhall, Chesire). Immobilised pH-gradient (IPG)-strips of pH 3–10 and IPG buffer of pH 3–10 were obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Primary antibodies were obtained from Novacastra Laboratories, Newcastle upon Tyne, UK (mAb DYS-2 to the carboxy-terminus of the dystrophin isoform Dp427), Affinity Bioreagents, Golden, CO, USA (mAb XIIC4 to sarcalumenin and pAb to cardiac calsequestrin), Sigma Chemical Company, Poole, Dorset, UK (pAb to laminin), and Upstate Biotechnology, Lake Placid, NY, USA (mAb C464.6 to the α_1-subunit of the Na^+/K^+-ATPase and mAb $VIA4_1$ to α-dystroglycan). Antibodies to the histine-rich Ca^{2+}-binding protein HRCBP (histidine-rich calcium binding protein) were a generous gift from Dr. Sandra L. Hofmann (Human Center for Therapeutic Oncology Research, Dallas, TX, USA). Peroxidase-conjugated secondary antibodies were purchased from Chemicon International (Temecula, CA, USA). Ultrapure Protogel acrylamide stock solutions were obtained from National Diagnostics (Atlanta, GA, USA). Protran nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany). All other chemicals used were of analytical grade and purchased from Sigma.

2.2. Preparation of cardiac membrane vesicles

All preparative steps were performed at 0–4 °C and all isolation buffers contained a mixture of protease inhibitors (0.2 mM Pefabloc, 1.4 μM pepstatin A, 0.3 μM E-64, 1 μM leupeptin, 1 mM EDTA, 0.5 μM soybean trypsin inhibitor). Hearts from 8- and 18-week-old normal control C57BL/10 mice and age-matched mdx mice of the Dmd^{mdx} strain (Jackson Laboratory, Bar Harbor, Maine, USA) were obtained through the Biomedical Facility of the National University of Ireland, Dublin. For 1D immunoblotting, a crude membrane fraction was prepared from normal and dystrophic heart, as well as skeletal muscle specimens, by a previously established protocol [24]. Membranes were resuspended at a protein concentration of 10 mg/ml and used immediately for gel electrophoretic separation.

2.3. Immunoblotting

For standard 1D gel electrophoresis and second-dimension separation, a Mini-MP3 electrophoresis system from Bio-Rad Laboratories (Hempel Hempstead, Hertsfortshire, Herts, UK) was employed. The gel electrophoretic separation of microsomal membrane proteins was carried out under reducing conditions in the presence of sodium dodecyl sulfate [25], using 7% (w/v) polyacrylamide gels run for 280 Vh with 25 μg protein per lane. Subsequent immunoblotting was performed according to Towbin et al. [26] using a Bio-Rad Mini-MP3 blotting cell system (Bio-Rad Laboratories). Antibody incubation and visualization of immuno-decorated protein bands by enhanced chemiluminescence (ECL) was carried out as previously described in detail [27]. Densitometric scanning of ECL-blots was performed on a Molecular Dynamics 300S computing densitometer (Sunyvale, CA) using ImageQuant V3.0 software. Statistical analysis

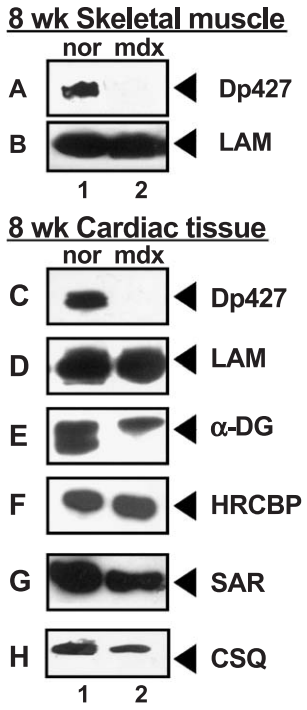

Fig. 1. Comparative one-dimensional immunoblot analysis of normal and dystrophic mdx skeletal muscle and heart membranes. Shown are immunoblots of normal (lane 1) and dystrophic mdx (lane 2) membranes isolated from skeletal muscle (A, B) or heart (C–H) homogenates. Blots were labelled with antibodies to the Dp427 isoform of dystrophin (A, C), laminin (LAM) (B, D), α-dystroglycan (α-DG) (E), the histidine-rich Ca^{2+}-binding protein (HRCBP) (F), sarcalumenin (SAR) (G) and calsequestrin (CSQ) (H). The position of immuno-decorated bands is marked by arrowheads.

using Graphpad Prism 4.01 software (Graphpad Software, San Diego, CA, USA) was used to perform *t*-test for unpaired observations.

2.4. 2D-electrophoretic analysis of total cardiac extracts

Prior to isoelectric focusing, whole hearts were ground to a fine powder using liquid nitrogen and subsequently solubilized in lysis buffer (9.5 M urea, 4% w/v CHAPS, 0.5% v/v carrier ampholytes 3–10, 100 mM DTT). The solution was supplemented with a freshly prepared protease inhibitor cocktail (0.2 mM Pefabloc, 1.4 μM pepstatin, 0.15 μM aprotinin, 0.3 μM E-64, 1 μM leupeptin, 0.5 mM soybean trypsin inhibitor and 1 mM EDTA) in order to prevent protein degradation. In addition, to reduce excessive viscosity of the extract due to DNA, 2 μl of DNase I (200 units) was added per 100-μl lysis buffer. After incubation for 3 h at 20 °C, the suspension was centrifuged for 20 min at 20,000 × *g*, and the supernatant diluted in lysis buffer to achieve a final protein concentration of 50 μg protein per strip for silver staining or subsequent immunoblotting experiments. Isoelectric focusing and separation in the second dimension was perfomed by established techniques [13]. Immunoblotting and silver staining of 2D gels was carried out as previously described in detail [28].

3. Results

3.1. Characterisation of cardiac mdx membranes

Prior to the 2D gel electrophoretic and immunoblot analysis of total cardiac extracts from normal and mdx tissues, the mutant status of the animal model was evaluated. In contrast to laminin (Fig. 1B and D), the Dp427 isoform of both skeletal and cardiac muscle dystrophin was found to be absent from mdx microsomal membrane preparations (Fig. 1A and C). As illustrated in Fig. 1E, the expression of a representative of the dystrophin-associated glycoprotein complex, α-dystroglycan, was drastically reduced in dystrophic mdx cardiac preparations. The initial 1D immunoblotting of luminal Ca^{2+}-regulatory proteins revealed on the one hand a potential increase in the histidine-rich Ca^{2+}-binding protein HRCBP, and on the

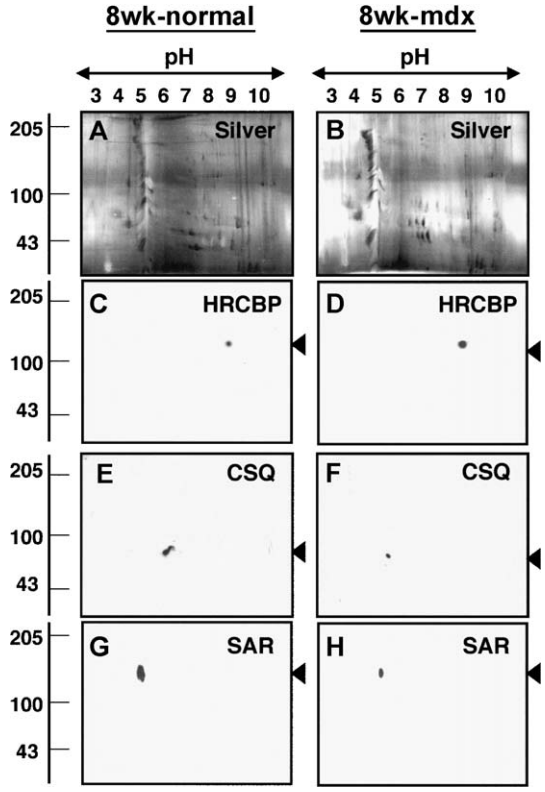

Fig. 2. Two-dimensional immunoblot analysis of luminal Ca^{2+}-binding proteins in 8 week old normal and mdx heart. Shown are silver-stained gels (A, B) and corresponding immunoblots (C–H) of total extracts from 8-week (wk)-old normal (A, C, E, G) and dystrophic mdx (B, D, F, H) cardiac muscle. Immunoblots were labelled with antibodies to the histidine-rich Ca^{2+}-binding protein (HRCBP) (C, D), calsequestrin (CSQ) (E, F) and sarcalumenin (SAR) (G, H). The position of immuno-decorated protein dots is marked by arrowheads. The pH values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated on the top and on the left of the panels, respectively.

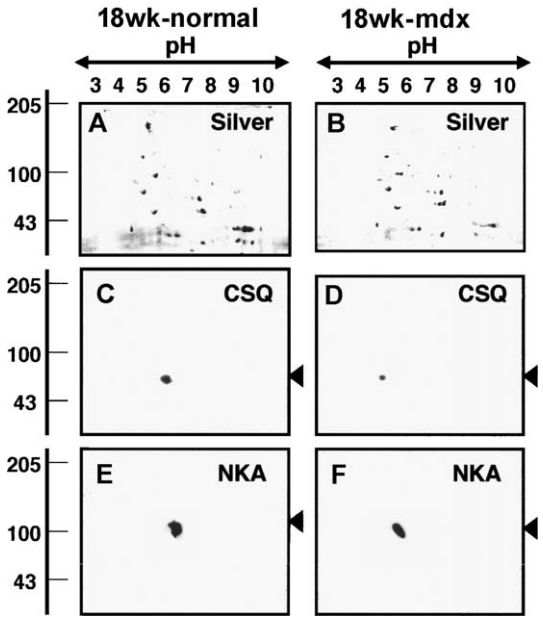

Fig. 3. Two-dimensional immunoblot analysis of calsequestrin in 18-week-old normal and mdx heart. Shown are silver-stained gels (A, B) and corresponding immunoblots (C–F) of total extracts from 18-week (wk)-old normal (A, C, E) and dystrophic mdx (B, D, F) cardiac muscle. Immunoblots were labelled with antibodies to calsequestrin (CSQ) (C, D) and the α_1-subunit of the Na^+/K^+-ATPase (NKA) (E, F). The position of immuno-decorated protein dots is marked by arrowheads. The pH values of the first dimension gel system and molecular mass standards (in kDa) of the second dimension are indicated on the top and on the left of the panels, respectively.

other hand a decrease in the amount of the terminal cisternae constituent CSQ and the Ca^{2+}-shuttle element SAR (Fig. 1F–H).

3.2. 2D immunoblot analysis of luminal Ca^{2+}-binding proteins in cardiac mdx tissue

For the comparative analysis of total cardiac extracts, we employed a 2D immunoblotting approach in this study. As illustrated in Fig. 2, the luminal Ca^{2+}-binding proteins HRCBP, CSQ and SAR exhibited distinct differences in their expression profile in 8-week-old dystrophic hearts as compared to age-matched normal cardiac tissue. In contrast, the silver staining pattern of 2D-separated cardiac muscle proteins did not reveal dramatic changes in the overall expression of abundant protein species (Fig. 2A and B). Certain spots of low density showed a modified intensity in diseased preparations versus normal controls. A future proteomics-based approach using mass spectroscopical identification methodology might be useful in the evaluation of these global changes in protein expression. However, current proteomic tools often miss the proper identification of integral proteins, very large proteins, aggregated assemblies, components with a very basic p*I*-value and/or proteins of low abundance. The background staining due to highly abundant proteins, such as myosin and actin in muscle preparations, can also diminish the accuracy of the mass spectroscopic analysis. This still allows immunoblotting with highly specific antibodies a central place in the list of reliable and versatile biochemical detection techniques. Interestingly, our immunoblotting approach detected molecular species by antibody decoration that did not correlate with distinct silver-stained dots in the 2D gel (Fig. 2C–H). While HRCBP exhibited a basic p*I*-value of approximately pH 8 to 9, the two other luminal Ca^{2+}-binding proteins showed a more acid p*I*-value in the pH 4 to 6 range. The relative molecular masses of the three immuno-decorated protein dots were approximately 130, 63 and 160 kDa for HRCBP, CSQ and SAR, respectively. Immuno-decoration clearly showed an increase in HRCBP expression and a decreased density of CSQ and SAR in dystrophic mdx heart preparations as compared to dystrophin-containing controls. Similar findings were obtained from older mdx heart preparations. The representative immunoblot analysis of cardiac CSQ in Fig. 3C and D demonstrates the reduction of this terminal cisternae marker in 18-week-old

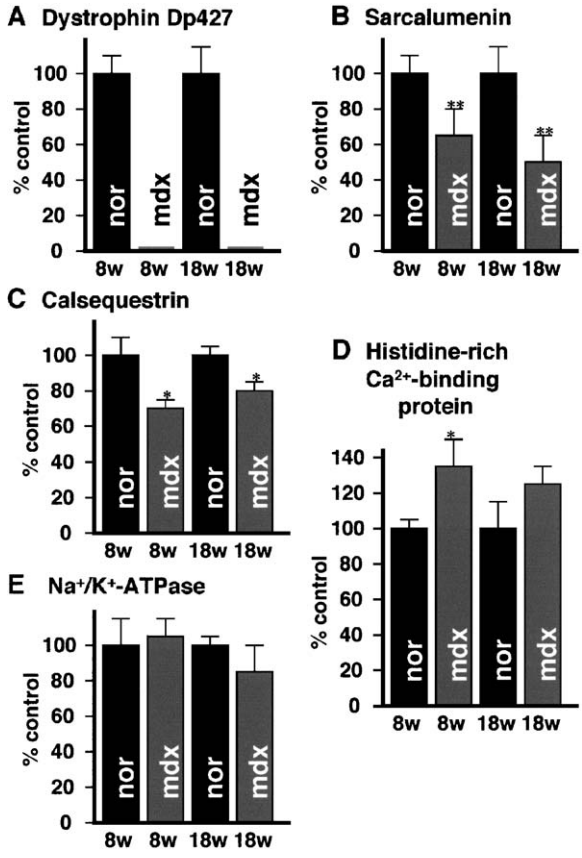

Fig. 4. Changed expression profiles of luminal Ca^{2+}-binding proteins in the dystrophic mdx heart. Shown is the graphical presentation of the immunoblot analysis (means ± S.D.; $n=5$; $*P<0.05$; $**P<0.01$) of 8- and 18-week-old normal and dystrophic mdx cardiac preparations. Panels A to E represent the dystrophin isoform Dp427, sarcalumenin, calsequestrin, the histidine-rich Ca^{2+}-binding protein and the Na^+/K^+-ATPase, respectively.

dystrophic preparations. The relatively comparable silver-staining pattern of Fig. 3A and B underlines the importance of this finding. Deficiency in dystrophin does not seem to cause a massive alteration in the expression profile of abundant cardiac proteins but has distinct effects on a subset of components. For control purposes and to show that equal loading conditions were employed, the comparative expression of the Na^+/K^+-ATPase is shown in Fig. 3E and F. Apparently, this surface ion pump is not drastically affected in its abundance in dystrophic heart.

3.3. Reduced calsequestrin and sarcalumenin expression in dystrophic heart muscle

The graphical presentation of our immunoblot analysis of luminal SR Ca^{2+}-binding proteins summarises the major findings of this report. In both mdx age groups studied, the deficiency in cardiac dystrophin (Fig. 4A) clearly resulted in a reduced expression of SAR (Fig. 4B) and CSQ (Fig. 4C) to a varying degree. In contrast, the HRCBP was increased in both 8- and 18-week-old dystrophic hearts (Fig. 4D). Immunoblotting of younger heart muscle specimens did not result in sufficient immuno-decoration above background staining (not shown) and was therefore not further pursued. The expression levels of the surface Na^+/K^+-ATPase were not drastically changed in the mdx heart preparations as compared to normal age-matched hearts (Fig. 4E).

4. Discussion

Cardiomyopathy is a serious complication associated with DMD [16,17]. Approximately 20% of deaths in X-linked muscular dystrophy are due to cardiac involvement [2]. Previous investigations into the degenerative pathways leading to skeletal muscle necrosis strongly indicate that, following sarcolemmal micro-rupturing, downstream alteration in Ca^{2+}-cycling plays a major role in muscular dystrophy [9,12–14]. Thus, although the primary abnormality in the full-length dystrophin isoform Dp427 is clearly the underlying cause for DMD [4], abnormal Ca^{2+}-fluxes represent the key pathophysiological process leading to the end-stage muscle wasting mechanism [8]. In analogy, here we can show that deficiency in the cardiac Dp427 protein also has an effect on Ca^{2+}-regulatory elements involved in the excitation–contraction–relaxation cycle of the heart. The fact that CSQ and SAR are important luminal mediators between the energy-dependent Ca^{2+}-uptake via Ca^{2+}-ATPase pump units and the Ca^{2+}-efflux mechanism through the junctional ryanodine receptor complex [29,30] makes the decreased abundance in the dystrophic heart a significant finding. It is important to stress that SR Ca^{2+}-binding proteins do not simply represent ion traps that facilitate the Ca^{2+} re-uptake against a less steep gradient and drastically increase the luminal Ca^{2+}-storage capacity, but were also shown to act as endogenous regulators of SR Ca^{2+}-channels [31]. Hence,

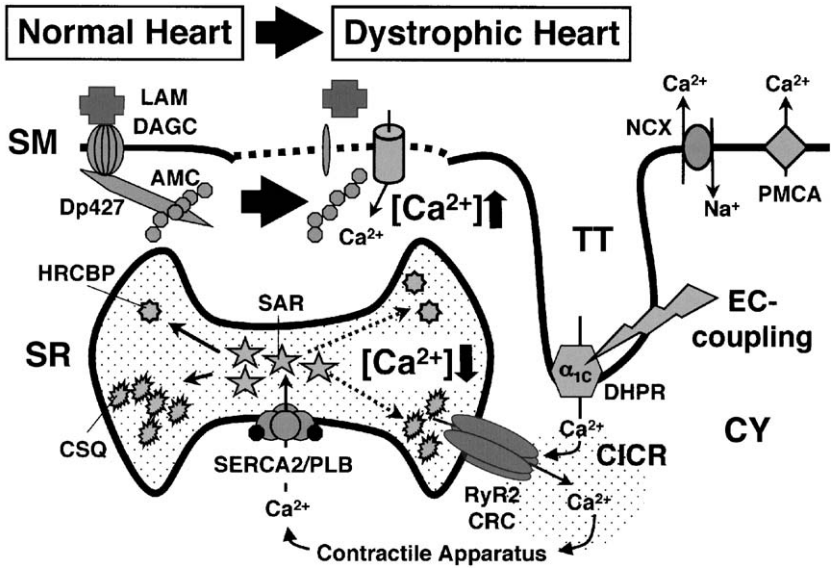

Fig. 5. Overview of abnormal Ca^{2+}-handling in the dystrophic heart. Shown is a diagram of the major Ca^{2+}-regulatory elements involved in cardiac excitation–contraction (EC) coupling and fibre relaxation. This includes the transverse-tubular (TT) α_{1C}-dihydropyridine receptor (DHPR) Ca^{2+}-channel and the RyR2 isoform of the sarcoplasmic reticulum (SR) ryanodine receptor Ca^{2+}-release channel (CRC), whose signal transduction mechanism is mediated by a Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism. Shown are the luminal Ca^{2+}-binding proteins sarcalumenin (SAR), calsequestrin (CSQ) and the histidine-rich Ca^{2+}-binding protein (HRCBP), the surface Ca^{2+}-removal system consisting of the Na^+/Ca^{2+}-exchanger (NCX) and the plasmalemma Ca^{2+}-ATPase (PMCA), as well as the SR Ca^{2+}-removal system represented by SERCA2 Ca^{2+}-ATPase units and its regulator subunit phospholamban (PLB). The effect of a primary deficiency in the Dp427 isoform of dystrophin on the dystrophin-associated glycoprotein complex (DAGC) of the cardiac surface membrane (SM), the extracellular matrix component laminin (LAM) and the actin membrane cytoskeleton (AMC) in the cytosol (CY) is indicated. The proposed pathophysiological increase in the cytosolic Ca^{2+}-levels and the decrease in luminal SR Ca^{2+}-buffering capacity are marked by solid arrows.

even relatively small changes in the expression levels of CSQ and SAR may trigger a considerable disturbance of the Ca^{2+}-cycling apparatus in the dystrophic heart, as outlined in Fig. 5.

For bulk Ca^{2+}-ion buffering, CSQ clusters are the most important luminal binding protein [32]. Although CSQ has a relatively low affinity for Ca^{2+}-ions with a dissociation constant of approximately 1 mM, the binding capacity of over 40 Ca^{2+}-ions per monomeric protein chain establishes CSQ as a high-capacity ion-binding protein [32,33]. The reduced expression in the dystrophic heart might have an effect on the overall Ca^{2+}-binding capacity of the cardiac SR, since CSQ oligomers exhibit positive co-operativity with respect to high capacity Ca^{2+}-binding [34]. Protein–protein interactions seem to play a central role in regulating luminal SR Ca^{2+}-levels. Since the luminal Ca^{2+}-concentration has a considerable influence on Ca^{2+}-release channel opening, CSQ clustering probably directly modifies Ca^{2+}-flux rates from the SR lumen [31]. Reduced CSQ expression, in conjunction with the recently reported decrease in the Ca^{2+}-release channel in the mdx heart [35], indicates that both Ca^{2+}-storage and Ca^{2+}-release are impaired in the dystrophic heart.

Increased expression of HRCBP [36] probably represents a compensatory mechanism [37]. In analogy to CSQ, cycles of phosphorylation and dephosphorylation of HRCBP are also implicated in modulating the activity of the junctional ryanodine receptor Ca^{2+}-release channel complex [38]. Up-regulation of this endogenous physiological regulator of Ca^{2+}-fluxing might counteract some of the loss in other Ca^{2+}-regulatory elements. In contrast, the reduced SAR expression may trigger impaired luminal ion transport. SAR co-localises with the SR Ca^{2+}-ATPase [29] indicating a mediating role between Ca^{2+}-uptake via the longitudinal tubules/terminal cisternae region and the Ca^{2+}-storage/release sites close to the junctional SR membrane. Hence, reduction in SAR most likely plays a major role in the progressive molecular pathogenesis of the cardiac involvement in X-linked muscular dystrophy.

Although major differences exist in the fine regulation of excitation–contraction coupling and fibre relaxation between skeletal muscle and heart [39], abnormal Ca^{2+}-cycling might underlie dystrophic changes in both types of muscle. It is clearly established that cardiac excitation–contraction coupling is based on a Ca^{2+}-induced Ca^{2+}-release mechanism [40], while skeletal muscle signal transduction at the triadic junction is mediated by direct receptor coupling [41]. Muscle relaxation in fast-twitching skeletal muscle fibres involves almost exclusively sarcolipin-regulated SERCA1 units of the SR Ca^{2+}-ATPase [42]. In contrast, cardiac relaxation is initiated by the activity of the surface Na^{+}/Ca^{2+}-exchanger and phospolamban-regulated SERCA2 complexes of the SR [42]. Hence, individual steps in Ca^{2+}-mediated fibre degeneration might show differences between the heart and skeletal muscles, but the general effect of elevated cytosolic Ca^{2+}-levels and decreased luminal Ca^{2+}-buffering appears to be similar in both classes of muscle tissue.

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