Ultrastructural remodeling of fast skeletal muscle fibers induced by invalidation of creatine kinase

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MATERIALS AND METHODS

Muscle Preparation

Engineered mice were a kind gift from Drs. B. Wieringa and F. Oerlemans (University of Nijmegen, Nijmegen, The Netherlands). Procedures involved in the generation and genotyping of the MM-CK and mi-CK null (CK−/−) mice have been described in detail elsewhere (24, 26). Adult (3–5 mo old) C57BL wild-type (WT) and CK−/− (mouse) and extensively

CREATINE KINASE (CK) is now recognized as a key player in transport of energy from the mitochondrion and within the cytoplasm, and in its utilization at the sites of consumption (23). The family of CK isoenzymes catalyzes the reversible transfer of a phosphate moiety between creatine and ATP. The major part of muscle CK exists (33) as the cytosolic isoenzyme (MM-CK) and as the mitochondrial isoenzyme (mi-CK). Part of MM-CK is structurally associated with myofibrils and membranes of the sarcoplasmic reticulum and functionally coupled to ATPases for optimal function of the contractile machinery and sarcoplasmic reticulum calcium uptake (18, 22, 29, 31).

Transgenic mice represent useful models for studies of effects of specific proteins in cellular functions. Recently, an engineered mouse with invalided expression of both mi-CK and MM-CK was developed (CK−/− mouse) and extensively studied (24–26) to understand the effects of altered energy metabolism. Functional tests revealed that the fast skeletal muscle of CK−/− mice has abnormal calcium transient and lacks burst activity at the onset of stimulation but exhibits paradoxical decreased fatigability (4, 6, 10, 24). Steeghs et al. (24) reported that in CK−/− mice, the fast glycolytic skeletal muscle fibers of gastrocnemius muscle show two new distinct ultrastructural features. First, specifically in males, CK−/− muscle fibers develop membranous inclusions called tubular aggregates (24) that react to both mitochondrial and sarcoplasmic reticulum markers (19). Second, more generally, was a marked increase in the relative mitochondrial mass (24). Proteomic and mRNA analysis (7, 8) as well as functional studies (14) confirmed the metabolic remodeling of the CK−/− gastrocnemius muscle toward a more oxidative phenotype. Moreover, these changes were accompanied by facilitation of direct functional interaction between mitochondria and other organelles (14) and by increased participation of other phosphotransfer systems (7, 25) that could both participate in the rescue of energy fluxes, suggesting changes in their physical interactions. It can be hypothesized that manipulating energy transport in muscle cells could also have an influence on the cytoarchitecture, from either an adaptational or a pathological point of view.

Thus, to come to specific conclusions, the morphologically observable remodeling needs to be supported by quantitative analysis to estimate its extent and to avoid subjective interpretation of its possible functional relevance. In the present study, we focused on the mitochondrial network in fast-twitch muscle fibers because it underwent profound remodeling and because of the unique role of these organelles in muscle energetics. The aim of this study was thus to characterize the changes in the ultrastructure in the fast-twitch muscle fibers of CK−/− mouse using a stereological approach. To achieve this aim, we introduced a new stereological parameter, the organelle environment, which was instrumental in characterizing changes of muscle cytoarchitecture.

MATERIALS AND METHODS

Muscle Preparation

Engineered mice were a kind gift from Drs. B. Wieringa and F. Oerlemans (University of Nijmegen, Nijmegen, The Netherlands). Procedures involved in the generation and genotyping of the MM-CK and mi-CK null (CK−/−) mice have been described in detail elsewhere (24, 26). Adult (3–5 mo old) C57BL wild-type (WT) and CK−/− (mouse) and extensively

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CK\(^{-/-}\) mice were anesthetized with an intraperitoneal Pentothal injection and killed. This investigation was conducted in accordance with our institutional guidelines defined by the European Community guiding principles in the care and use of animals and French decree no. 87/848 of October 19, 1987. Authorizations to perform animal experiments according to this decree were obtained from the French Ministry of Agriculture and Fisheries (no. 7474, May 2, 2002).

Gastrocnemius muscles were isolated from five WT and five CK\(^{-/-}\) mice. Dissected muscles were placed in nominally calcium-free Krebs solution for 10 min, stretched by 30% of their resting length, and fixed with 2% glutaraldehyde in cacodylate buffer (in mmol/l: 150 Na-cacodylate, 2.0 CaCl\(_2\)) at pH 7.3 for 45 min. After fixation, five samples of each muscle were dissected from the randomly selected superficial regions around the central part of the muscle. Samples were then placed for 30 min in cacodylate buffer with 2% glutaraldehyde, washed with cacodylate buffer, post-fixed for 30 min in cacodylate buffer with 1% OsO\(_4\), and contrasted with 2% uranyl acetate in aqueous solution. After gradual dehydration in ethanol, the samples were embedded through acetone in Durcupan (Fluka, Geneva, Switzerland). Ultrathin (60–80 nm) longitudinal sections were cut using a Porter-Blum-MT-2 ultramicrotome, placed on 400-mesh copper grids covered with formvar, and contrasted with lead citrate (21). The sections were examined with a JEM 1200 electron microscope (Jeol, Tokyo, Japan). Micrographs were taken on 9-cm Scientia EM films (Agfa, Mortsel, Belgium).

Stereological Analysis

Three longitudinal sections were cut from each tissue block at three randomly selected levels separated by more than 50 μm. To select randomly the region of the fiber for stereological analysis, the windows of the mesh grid that laid above the central part of the section were photographed. Three negatives from each section were obtained, which represented random areas across the diameter of the fiber. Micrographs taken at a magnification of ×12,000 were digitized at 600 dpi resolution, converted to positives, balanced uniformly by using the contrast and brightness controls in Adobe Photoshop (version 6.0; Adobe Systems), and analyzed using a personal computer (133 MHz Pentium, 32 MB RAM, 2 MB Matrix Millennium graphics card, 15-inch monitor display screen) equipped with ImageTool (version 2.0; University of Texas Health Sciences Center, San Antonio, TX) and Adobe Photoshop software.

The stereological analysis was performed using test grids composed of test points and of cycloid curves. The test grid was generated and properly overlaid over the image by using STESYS software (15) running under Microsoft Windows as a module of the ImageTool system. The test grid was composed of 532 test points separated by 257 nm. The length of the cycloids was 440 nm. Altogether, 4,239 μm\(^2\) of 62 fibers of the WT mice group and 4,650 μm\(^2\) of 75 fibers from the CK\(^{-/-}\) mice group were evaluated. The final image magnification on the computer screen during analysis was ×72,000.

Estimation of Volume and Surface Density

In this study we focused on organelles important for the contractile function of muscle fibers. These include mitochondria, myofilibrils, sarcoplasmic reticulum, and transverse tubules. Most of these organelles were analyzed in more detail. In myofilibrils, the Z lines, I bands, and A bands, and in sarcoplasmic reticulum, the terminal cisterns and free SR were evaluated. Mitochondria were analyzed as subsarcolemmal, intermyofibrillar, and modified mitochondria. Subsarcolemmal mitochondria were identified as those occurring between the sarcolemma and the first layer of myofilibrils. Intermyofibrillar mitochondria were identified as those occurring between myofilibrils. Modified mitochondria were identified as those containing homogenous material with modified cristae or vacuoles. Transverse tubules were identified near the I/A band boundary, where they appeared as empty, membrane-delimited tubular profiles bound with terminal cisterns. Tubular aggregates and modified mitochondria were analyzed in CK\(^{-/-}\) fibers only, because they did not occur in WT fibers. The free cytoplasm, that is, the intracellular milieu outside of organelles, completes the list of evaluated entities.

The volume density, \(V_v\), of cellular components was estimated using a point integration method according to Bolender and Weibel (3) as \(V_v = pLP\), where \(p\) is the number of test points hitting the image of the cellular component of interest in the evaluated area of the fiber and \(P\) is the number of all points falling on the image of the muscle fiber.

The surface density, \(S_v\), of cellular components was estimated using the methods of vertical sections according to Baddeley et al. (2), which employs cycloid curves instead of lines to compensate for errors due to preferential longitudinal organization of intracellular structures in skeletal muscle cells. The surface density was calculated as \(S_v = 2NiLP\), where \(N\) is the number of test points in the grid, \(i\) is the number of intersections between the cycloids and the outer edge of the cellular component of interest, \(L\) is the overall length of cycloids in the applied test grid, and \(P\) is the number of the test points that hit the cell.

Estimation of Mitochondrial Environment

Environment of mitochondria, \(E\), was estimated using the relation \(E = I_sI_l\), where \(I_s\) is the number of intersections of cycloids with the outer edge of the cellular component of interest, which was in the vicinity of a mitochondrion at a distance of 20 nm or less, and \(I_l\) is the number of intersections of cycloids with the outer edge of mitochondria.

The data analysis was performed using Origin (version 5.0; Microcal). Results are expressed as means ± SE. The statistical significance of differences between groups was estimated using one-way ANOVA and Student’s \(t\)-test. The difference was considered as statistically significant at the level of 0.05.

RESULTS

Ultrastructure of WT and CK\(^{-/-}\) Fast Muscle Fibers

Muscle fibers of WT mice (Fig. 1A) displayed typical ultrastructural features of fast glycolytic fibers. Myofilibrils were arranged in register, Z lines were very thin, transverse tubules formed triads with cisterns of sarcoplasmic reticulum near the I/A band boundaries, nuclei were under sarcolemma, and the apparent content of mitochondria was low. Profiles of intermyofibrillar mitochondria in sections were small, isolated from each other, and localized at sides of I bands near Z lines (Fig. 1A). Subsarcolemmal mitochondria were similar to intermyofibrillar mitochondria except in the perinuclear regions, where they formed small clusters around nuclei. Ultrastructural appearance of the WT fibers was homogenous over their whole length.

In CK\(^{-/-}\) mice, the ultrastructure of muscle fibers was not homogenous. Regions with distorted ultrastructure were observed all along the fiber volume in addition to regions indistinguishable from WT muscle fibers (Fig. 1, B–D). Interfilibrillar mitochondria varied in size and were more abundant than in WT fibers (Fig. 1, B–D). They often spanned the whole sarcomere length. Subsarcolemmal mitochondria were very heterogeneous in size and shape and formed multilayered clusters (Fig. 1D). In the altered regions, myofilibrils were not arranged in register and often were split so that much thinner myofilament bundles were observed (Fig. 1E). Tubular aggregates of various size emerged between myofilibrils and under sarcolemma (Fig. 1F). At the periphery of tubular aggregates,
unusual mitochondria often containing electron-dense material with remnants of cristae and showing signs of damage (vacuolization) were frequently observed (Fig. 1, D and F). Occasional myosin-like inclusions were observed in modified mitochondria.

Volume density. Generally, differences in the relative content of individual constituents between the WT and CK−/− fibers of gastrocnemius muscle were remarkable. Volume densities of evaluated structures are summarized in Table 1. Compared with WT fibers, the volume density of the overall mitochondrial population in CK−/− fibers was found nearly two times larger. The subsarcolemmal mitochondria occupied a 2.6 times larger and the intermyofibrillar population a 1.6 times larger portion of the cell volume. A small population of modified mitochondria observed at the periphery of tubular aggregates in CK−/− fibers participated by only 0.5% to the fiber volume but represented 5% of all mitochondria in CK−/− fibers. Tubular aggregates occupied ~5% of the CK−/− fiber volume. This value is comparable to the volume fraction of other membranous organelles or the free cytoplasm. The volume density of the free sarcoplasmic reticulum was the same in both muscle groups, but the content of terminal cisterns was significantly higher (1.2 times) in the CK−/− fibers. The overall volume density of membranous structures (mitochondria + sarcoplasmic reticulum + terminal cisterns + transverse tubules + tubular aggregates) was 1.7 times higher in CK−/− than in WT fibers. Interestingly, the volume fraction of the free cytosol was also significantly higher (1.9 times) in CK−/− than in WT muscle fibers. Volume analyses revealed significantly lower (1.17 times) volume density of myofibrils in CK−/− relative to WT mice. Considering the cylindrical shape of myofibrils, this could result from the reduction of the number of myofibrils in the unit volume of the fiber and/or from the reduction of their diameter. Significantly higher (+18%) surface-to-volume ratio of myofibrils in CK−/− than in WT fibers (Table 1) suggests that reduction of their diameter may be the significant factor.

Changes in the ultrastructure could lead to changes in relative proportions between organelles. Indeed, per Table 1, volume density ratios of mitochondria to myofibrils and of mitochondria to entire sarcoplasmic reticulum were substantially higher (2 times) in CK−/− than in WT fibers. At the same
time, however, volume density ratios of the sarcoplasmic reticulum to myofibrils and of the transverse tubules to terminal cisterns were not changed. This unchanged ratio, in addition to its functional consequences, indicates good quality of stereological analysis, because morphological analysis of triads in WT and CK−/− fibers did not reveal any changes in the form of these small and infrequent structures.

Surface densities. Analyses of the surface density give an insight into the surface area of cellular structures related to the cell volume. The surface density data in Table 1 show that substantial changes due to lack of CK occurred only in the surface density of mitochondria, whereas differences in surface density of other organelles were neither substantially nor statistically significant. The overall surface density of mitochondria was ~1.3 times higher in CK−/− than in WT mice, mainly due to substantially higher (2.4 times) surface density of the subsarcolemmal mitochondria. Interestingly, the surface-to-volume ratio (Table 1) of subsarcolemmal mitochondria did not change, suggesting that the number of subsarcolemmal mitochondria is higher in CK−/− fibers. On the other hand, significant decrease in the surface-to-volume ratios of intermyofibrillar mitochondria suggests that their size is 1.4 times larger in CK−/− than in WT fibers.

The surface density of the outer surface area of tubular aggregates in CK−/− fibers was very small, obviously due to their voluminous shape, as reflected by their very low surface-to-volume ratio (Table 1). Surface densities, as well as the surface-to-volume ratio, of sarcoplasmic reticulum and transverse tubules in CK−/− fibers were close to their respective values in WT fibers. A change in the volume density of terminal cisterns was not accompanied by a significant change of the surface density or the surface-to-volume ratio.

Interestingly, in contrast to their much higher overall volume density, the overall surface density of membranous organelles in CK−/− fibers was not statistically different from that in WT fibers, despite development of tubular aggregates. It should be noted, however, that the surface density of tubular aggregates was analyzed as an effective area of its abstract envelope, but considering the membranous character of individual tubules in the aggregates would considerably increase the overall membranous surface area.

Surface density values of myofibrils and their components in the CK−/− fibers did not follow the decrease in their volume density values but were the same in both groups. As was the case with the volume densities, surface density ratios of mitochondria to myofibrils and of mitochondria to entire sarcoplasmic reticulum were substantially higher (1.4 times) in CK−/− than in WT fibers. At the same time, surface density ratios of the sarcoplasmic reticulum to myofibrils and of the transverse tubules to terminal cisterns were unchanged.

Mitochondrial environment: CK−/− vs. WT. The lack of creatine kinase substantially changed the overall mitochondrial population of gastrocnemius muscle fibers. However, the two mitochondrial populations changed differently. Therefore, the environment of subsarcolemmal and intermyofibrillar mitochondria was also evaluated separately (Table 2).

The surface of subsarcolemmal mitochondria facing the myofibrils was significantly smaller (0.65 times), whereas the surface facing neighboring subsarcolemmal mitochondria was significantly higher (1.79 times), in CK−/− than in WT fibers. These findings are in good agreement with morphological observations in CK−/− fibers that subsarcolemmal mitochondria show substantial grouping into clusters, usually near the nuclei. The relative contact area of subsarcolemmal mitochondria with membranous organelles appeared increased in CK−/− fibers, but the changes were not statistically significant.

In the case of the intermyofibrillar mitochondria, three differences with respect to WT were most conspicuous in

### Table 1. Volume densities, surface densities, and surface-to-volume ratios of different constituents of gastrocnemius muscle fibers of wild-type and CK−/− mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CK−/−</th>
<th>WT</th>
<th>CK−/−</th>
<th>Svv/Vv, × 10−1, μm−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM</td>
<td>0.96±0.14</td>
<td>2.54±0.32</td>
<td>0.05±0.01</td>
<td>0.13±0.01</td>
<td>58.3 ± 52.7</td>
</tr>
<tr>
<td>IM</td>
<td>4.45±0.07</td>
<td>7.19±0.67</td>
<td>0.39±0.01</td>
<td>0.45±0.03</td>
<td>88.3 ± 62.6</td>
</tr>
<tr>
<td>MM</td>
<td>n.a.</td>
<td>0.50±0.13</td>
<td>n.a.</td>
<td>0.03±0.01</td>
<td>n.a. ± 50.4</td>
</tr>
<tr>
<td>MI</td>
<td>5.41±0.10</td>
<td>10.22±0.57</td>
<td>0.45±0.01</td>
<td>0.60±0.03</td>
<td>79.0 ± 59.3</td>
</tr>
<tr>
<td>ZL</td>
<td>1.73±0.08</td>
<td>1.82±0.09</td>
<td>0.06±0.00</td>
<td>0.07±0.00</td>
<td>35.2 ± 36.4</td>
</tr>
<tr>
<td>IB</td>
<td>23.27±1.22</td>
<td>20.75±0.88</td>
<td>0.65±0.06</td>
<td>0.62±0.03</td>
<td>27.7 ± 30.0</td>
</tr>
<tr>
<td>AB</td>
<td>57.45±1.07</td>
<td>48.14±0.94</td>
<td>0.82±0.09</td>
<td>0.86±0.05</td>
<td>14.1 ± 17.8</td>
</tr>
<tr>
<td>MF</td>
<td>82.45±0.69</td>
<td>70.71±1.04</td>
<td>1.53±0.06</td>
<td>1.54±0.07</td>
<td>18.5 ± 21.8</td>
</tr>
<tr>
<td>SR</td>
<td>7.08±0.52</td>
<td>6.55±0.51</td>
<td>1.33±0.10</td>
<td>1.16±0.03</td>
<td>187.6 ± 182.1</td>
</tr>
<tr>
<td>TC</td>
<td>1.86±0.09</td>
<td>2.23±0.06</td>
<td>0.56±0.03</td>
<td>0.65±0.04</td>
<td>304.1 ± 295.6</td>
</tr>
<tr>
<td>TT</td>
<td>0.49±0.05</td>
<td>0.58±0.06</td>
<td>0.24±0.01</td>
<td>0.28±0.02</td>
<td>497.6 ± 510.8</td>
</tr>
<tr>
<td>TA</td>
<td>n.a.</td>
<td>4.64±0.99</td>
<td>n.a.</td>
<td>0.06±0.01</td>
<td>n.a. ± 13.6</td>
</tr>
<tr>
<td>SL</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.07±0.01</td>
<td>0.06±0.01</td>
<td></td>
</tr>
<tr>
<td>CS</td>
<td>2.71±0.40</td>
<td>5.07±0.56*</td>
<td>n.a.</td>
<td>4.18±0.16</td>
<td>4.37±0.12</td>
</tr>
<tr>
<td>Σ</td>
<td>4.01±0.16</td>
<td>4.37±0.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MI/MF</td>
<td>0.07±0.00</td>
<td>0.15±0.01*</td>
<td>0.29±0.01</td>
<td>0.40±0.03*</td>
<td></td>
</tr>
<tr>
<td>SM/MiMF</td>
<td>0.11±0.01</td>
<td>0.12±0.01</td>
<td>1.24±0.03</td>
<td>1.18±0.03</td>
<td></td>
</tr>
<tr>
<td>MM/MMiMF</td>
<td>0.61±0.03</td>
<td>1.17±0.06*</td>
<td>0.24±0.01</td>
<td>0.33±0.02*</td>
<td></td>
</tr>
<tr>
<td>TT/TC</td>
<td>0.27±0.03</td>
<td>0.26±0.03</td>
<td>0.48±0.01</td>
<td>0.43±0.01</td>
<td></td>
</tr>
</tbody>
</table>

All values are presented as means ± SE; n = 5. *p < 0.05 compared with corresponding wild-type (WT) group. Vv, volume density; Svv, surface density; Svv/Vv, surface-to-volume ratio; SM, subsarcolemmal mitochondria; IM, intermyofibrillar mitochondria; MM, modified mitochondria; MI, total mitochondria; ZL, Z line; IB, I band; AB, A band; MF, total myofibrils; SR, sarcoplasmic reticulum; TC, terminal cisterns of sarcoplasmic reticulum; TT, transverse tubules; TA, tubular aggregates; SL, sarcolemma; CS, cytosol; Σ, sum of all cell constituents; SMiMF, sum of SM and MF; MMiMF, sum of MM and MF; MTT/TC, ratio of MM to TC; Svv/SM, surface density ratio of SM to MF.


Table 2. Environment of the subsarcolemmal and interfibrillar mitochondria of gastrocnemius muscle fibers of WT and CK<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>E, %</th>
<th>SM WT</th>
<th>SM CK&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>IM WT</th>
<th>IM CK&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM</td>
<td>16.89±3.05</td>
<td>29.76±2.28*</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>IM</td>
<td>n.a.</td>
<td>6.04±0.73</td>
<td>11.52±1.68*</td>
<td></td>
</tr>
<tr>
<td>MM</td>
<td>n.a.</td>
<td>0.39±0.33</td>
<td>n.a.</td>
<td>0.44±0.32</td>
</tr>
<tr>
<td>MI</td>
<td>16.89±3.05</td>
<td>30.16±3.06*</td>
<td>6.04±0.73</td>
<td>11.95±1.45*</td>
</tr>
<tr>
<td>ZL</td>
<td>1.45±0.45</td>
<td>0.48±0.24†</td>
<td>3.59±0.40</td>
<td>2.79±0.10†</td>
</tr>
<tr>
<td>IB</td>
<td>11.69±1.34</td>
<td>7.25±0.56*</td>
<td>4.66±2.29</td>
<td>26.63±1.45*</td>
</tr>
<tr>
<td>AB</td>
<td>17.92±2.98</td>
<td>12.54±1.22</td>
<td>8.22±1.55</td>
<td>24.85±2.35*</td>
</tr>
<tr>
<td>MF</td>
<td>31.06±2.25</td>
<td>20.27±1.46*</td>
<td>58.49±1.87</td>
<td>54.27±2.18</td>
</tr>
<tr>
<td>SR</td>
<td>2.28±0.66</td>
<td>3.06±0.50</td>
<td>11.88±1.79</td>
<td>8.66±1.44</td>
</tr>
<tr>
<td>TC</td>
<td>2.94±0.91</td>
<td>4.21±0.30</td>
<td>8.73±0.88</td>
<td>8.82±0.68</td>
</tr>
<tr>
<td>TT</td>
<td>0.16±0.16</td>
<td>0.57±0.31</td>
<td>0.63±0.25</td>
<td>0.86±0.17</td>
</tr>
<tr>
<td>TA</td>
<td>n.a.</td>
<td>2.51±2.24</td>
<td>n.a.</td>
<td>1.45±0.50</td>
</tr>
<tr>
<td>CS</td>
<td>26.42±4.74</td>
<td>22.92±2.29</td>
<td>14.23±1.48</td>
<td>14.00±1.30</td>
</tr>
<tr>
<td>SL</td>
<td>20.25±2.85</td>
<td>16.31±1.79</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

All values are presented as means ± SE; n = 5. E, environment of the mitochondria. *P < 0.05; †P < 0.1 compared with corresponding WT groups.

CK<sup>−/−</sup> fibers: about two times higher contact area with interfibrillar mitochondria, almost two times smaller contact area with the I band, and almost three times higher contact area with the A band. Such changes can be explained by extensive relocation of the interfibrillar mitochondria from their almost exclusive positioning at the side of I bands in the WT fibers toward the side of A bands in the CK<sup>−/−</sup> fibers. Definitely, increased frequency of interfibrillar mitochondria between A bands is evident in longitudinal sections of the CK<sup>−/−</sup> fibers. These changes are further supported by the increased size of interfibrillar mitochondria, reduced coverage of interfibrillar mitochondria by sarcoplasmic reticulum, and to some extent also by distortions in parallel arrangement of sarcomeres in the CK<sup>−/−</sup> fibers.

**DISCUSSION**

The aim of this study was to verify the hypothesis that specific impairment in energy transport recruits compensatory mechanisms that, in addition to molecular adaptation (14), lead to adaptation of the muscle cell microarchitecture. To achieve this, we applied a quantitative stereological approach to muscle fibers of the transgenic mouse model with knocked out mitochondrial and interfibrillar isoforms of CK. In addition to classic parameters, the volume and surface densities of cell constituents, we introduced a new parameter, the organelle environment, to measure changes in the neighborhood of mitochondria. We found that the microarchitecture of muscle cells adapted in line with other compensatory mechanisms toward sustaining the cell’s contractile function.

**General Changes**

Basic differences between WT and CK<sup>−/−</sup> fibers observed in our study correspond to previously reported differences (26, 25), but the details were even more interesting. Generally, the CK<sup>−/−</sup> fibers displayed higher content of mitochondria in both the subsarcolemmal and the interfibrillar regions, delocalization of mitochondria from the preferential I band proximity toward the A band proximity, lower content and reduced diameter of myofibrils, and the occurrence of tubular aggregates. It should be stressed, however, that these changes were not obvious all over the fiber volume. To assess their extent and importance, exact quantitative analysis of structural remodeling was necessary. In this study we focused on the mitochondrial population because of its importance in muscle energetics and because it underwent the most obvious transformation.

**Mitochondria**

The substantially higher volume density of mitochondria in CK<sup>−/−</sup> than in WT fibers may indicate an overall increase in the oxidative capacity of the tissue, supposing that functionality of mitochondria was not changed. Our data show that volume density ratios of mitochondria to myofibrils and to sarcoplasmic reticulum, the two most energy-demanding organelles, were about two times higher in the CK<sup>−/−</sup> than in the WT fibers. The increased volume of interfibrillar mitochondria has already been observed in the gastrocnemius muscle with only the cytosolic isofrom of CK disabled (26). A 1.5- to 2-fold increase in volume density of interfibrillar mitochondria in CK<sup>−/−</sup> muscle reported by Steeghs et al. (25) is in accordance with our results. This also fits well with the twofold increase in oxidative capacity measured in CK<sup>−/−</sup> muscle fibers (14).

Another important finding regarding mitochondria and the lack of CK in our study is that the neighborhood of mitochondria was substantially modified. This can be interpreted as changed propensity of mitochondria to nourish, exchange metabolites, or communicate with individual organelles or spaces directly. Intermyofibrillar mitochondria changed their neighborhood differently than subsarcolemmal mitochondria. Dissimilar reaction of the two mitochondrial populations may have different grounds, as indicated by the increased number of subsarcolemmal mitochondria but increased size of interfibrillar mitochondria.

**Subsarcolemmal mitochondria**. In absolute values, the surface density of subsarcolemmal mitochondria was almost 2.5 times higher in CK<sup>−/−</sup> than in WT fibers. At the same time, coverage of the subsarcolemmal mitochondria surface also changed substantially, but not due to contacts with newly formed tubular aggregates, which represented only a minor part. An especially large increase that resulted from formation of large clusters was found in the contact area between the subsarcolemmal mitochondria. The functional meaning of clustering is not clear, because in effect it leads to reduction of the effective mitochondrial area for exchange of metabolites and to development of accumulation/depletion effects in diffusion-restricted spaces. In fact, subsarcolemmal mitochondria themselves took over one-half of their surface area increase. Clustering of mitochondria seems to result from their local proliferation and not from coalescing or growth.

**Interfibrillar mitochondria**. Changes in the environment of the interfibrillar mitochondria of CK<sup>−/−</sup> fibers compared with WT fibers were even more striking than in the case of subsarcolemmal mitochondria. The most important was the substantial increase in the surface area of interfibrillar mitochondria facing the A bands and its significant decrease near the I bands. Obviously, it could not be due to the increased volume or surface density of interfibrillar mitochondria. In that case, we
would expect increased coverage of mitochondria with the I band as well. In fact, the interfibrillar mitochondria surface density was not increased significantly in CK−/− over the WT fibers, but, as was already pointed out, the size of interfibrillar mitochondria was considerably increased. Stereological data confirmed and put into numbers morphological observations that the small mitochondria, localized preferentially at I bands in the fast WT muscle fibers, are in CK−/− muscle fibers partially replaced by larger mitochondria localized preferentially along the A band.

The relation of interfibrillar mitochondria to sarcoplasmic reticulum and terminal cisterns was not significantly different in CK−/− and WT fibers, although the volume and surface densities of terminal cisterns in CK−/− fibers were slightly higher. Very likely, a subtle relocation of interfibrillar mitochondria from the zones of maximal occurrence of terminal cisterns and sarcoplasmic reticulum membranes can be partially responsible. Finally, there was also a substantial increase in the mutual contact area between the interfibrillar mitochondria in CK−/− fibers over the WT fibers, as was found for subsarcolemmal mitochondria.

We have pointed out already that the subsarcolemmal mitochondria and interfibrillar mitochondria react differently to invalidation of the CK systems. The specific reactions of subsarcolemmal mitochondria and interfibrillar mitochondria support previous biochemical and functional studies, which have established that in rat gastrocnemius muscle the subsarcolemmal mitochondria have lower oxidative capacity than interfibrillar mitochondria and respond differently to chronic changes in the level of contractile activity (17). Interestingly, endurance training also increased mitochondrial volume in the subsarcolemmal space twice as much as in the intermyofibrillar space (12).

Myofibrils

The higher surface-to-volume ratio of myofibrils in CK−/− than in WT fibers, due to reduction of the volume density and unchanged surface density of myofibrils, suggests a reduction of myofibrillar diameter as envisioned by Van Deursen et al. (26), who proposed that in this muscle, the diffusion distance between myofibrils and mitochondria was decreased. Such a change can be regarded as an adaptation improving supply of the contractile myofilaments with calcium and energy substrates resulting from the decreased diffusion distance (32, 30). From surface-to-volume density data, we can estimate a reduction of the average myofibril radius by 18%, equivalent to a reduction of the average fiber cross section by 40%. Moreover, there was a significant decrease of the relative myofibrillar volume from 82% to 71%. These numbers compare well with the 25% decrease in total myosin heavy chain protein content (7) as well as with the 32% decrease in maximal force (4) observed in single fibers from flexor digitorum brevis, another fast skeletal muscle, of CK−/− mice.

Sarcoplasmic Reticulum

Of interest is the statistically significant 20% increase of the volume density of terminal cisterns in CK−/− mice. This result confirms and extends the previous observation of terminal cistern proliferation (25) and indicates an increased capacity of CK−/− fibers for calcium storage and release during contraction. This is in accordance with slightly larger calcium transients in CK−/− than in WT fibers (5).

Cytosol

Overall volume of the cytosol represents a very small fraction (2.7%) of the cell volume in the WT gastrocnemius. The increased relative volume of cytosol in CK−/− fibers may be related to the increased volume of interfibrillar mitochondria and reduced diameter of myofibrils, resulting in less densely packed cell structure with freer cytoplasm around organelles.

Tubular Aggregates

The presence of tubular aggregates was encountered only in CK−/− muscle fibers as previously described (24). We have shown recently by means of an immunocytochemical approach that tubular aggregates are formed with participation of the inner mitochondrial membranes and membranes of the sarcoplasmic reticulum (19). At present, however, the functional significance of such tubular aggregates associated with mitochondrial structures is not known.

Possible Physiological Implications

Different studies have shown increased amount of mitochondrial enzymes and increased oxidative capacity in the fast CK−/− gastrocnemius muscle fibers, indicating that in contrast to mitochondrial diseases, mitochondria of CK−/− fibers are indeed functional (14, 30). Increased proximity of mitochondria to contractile fibrils, quantified in this study, can be considered as evidence for ultrastructural adaptation toward a direct energy support of contraction. Our results show that an increased role of energy transport via ADP and ATP molecules, proposed by Steeghs et al. (24), results from their substantially reduced diffusion distance and time. This conclusion is in line with the statement of de Groof et al. (8) that besides global proliferation of mitochondrial mass, there is architectural plasticity and fine regulation of the mitochondrial design in distinct CK-deficient mice. Direct energy cross talk between mitochondria on one hand and myofibrils or sarcoplasmic reticulum on the other has been demonstrated recently in highly oxidative cardiac muscle (13). It is striking to observe that this direct energetic interaction is totally absent in gastrocnemius muscle of WT mice and becomes highly functional in CK−/− mice. This opens the possibility that the mitochondria reorganized toward closer contacts with myofilaments, supporting direct channeling of ATP and ADP, and thus, together with increased activity of other phosphotransfer pathways, i.e., adenylate kinase and glycolytic systems (9), rescue relaxation of rigor tension and calcium uptake (14) in vitro provide fatigue resistance to the fast CK−/− skeletal muscle and allow for almost normal contractile activity in vivo.

Mitochondria of the CK-deficient muscles exhibit a lower sensitivity for cytosolic ADP, similar to that observed in oxidative muscles (27, 16, 14). It has been suggested that the sensitivity of muscle mitochondria to cytosolic ADP depends on the cytoarchitecture and cytoskeleton (1). Because the apparent Km for cytosolic ADP increased in CK−/− gastrocnemius muscle in parallel with increased crowding of mitochondria as demonstrated in this study, it is tempting to speculate that high Km for ADP in oxidative muscles is simply a direct consequence of mitochondria being crowded within...
clusters, leading to decreased access of cytosolic ADP to the mitochondrial surface. In conclusion, the mitochondrial reorganization is the structural adaptive mechanism representing an alternative system to the local control of adenine nucleotide pools.

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