Impaired muscular contractile performance and adenine nucleotide handling in creatine kinase-deficient mice

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Creatine kinase (CK) consists of a small family of isoenzymes catalyzing the transphosphorylation between phosphocreatine (PCr) and ATP (2, 19). Skeletal muscle contains two distinct isoforms, which are localized either in the cytosol (MM-CK) or at the inner site of the outer mitochondrial membrane (Miβ-CK) (2, 19, 23, 24). Both CK isoforms play an important role in maintaining muscular ATP and ADP concentrations, e.g., during strenuous exercise (3). Previously, it was shown that ATP levels are lower in resting skeletal muscle of mice with combined deficiency in MM-CK and Miβ-CK (14). Interestingly, the resting skeletal muscle PCr and creatine content was found to be comparable with that of age-matched control mice despite the virtual lack of CK activity in the former (4, 14). Moreover, the resting inosine monophosphate (IMP) level was enhanced in CK-deficient mice, which strongly suggests a disturbance in ATP regeneration (14).

Skeletal muscle of MM-CK- and Miβ-CK-deficient mice shows impaired contractile properties, which are marked by diminished tetanic force output and prolonged relaxation time (8, 14). The relationship between depressed contractility and utilization of high-energy phosphates in CK-deficient mice, however, has barely been investigated. In the present study, we explored the hypothesis that muscles lacking both CK isoforms are unable to properly handle their high-energy phosphate stores, which results in an impaired muscular functioning. To test this hypothesis, contractile performance during a single tetanic contraction of dorsal flexors of CK−/− mice was determined while keeping the muscle in its natural surroundings. Possible differences in contractile behavior during a single tetanic contraction between CK−/− and wild-type mice were related to differences in resting content of high-energy phosphates and associated compounds in the muscle group studied.

Furthermore, the rate of decline in maximal torque during a series of 12 repetitive isometric tetanic contractions was monitored. By analyzing the tissue content of high-energy phosphates and related compounds in resting muscle immediately after the series of tetanic contractions and after a recovery period of 60 s, we investigated the effect of the lack of CK activity on ATP regeneration in muscles subjected to this high-intensity exercise series.

Traditionally, mechanical functioning of muscles of small rodents is determined under less physiological in vitro conditions, e.g., isolated muscles with impaired innervation and blood supply, which may influence the mechanical properties of the muscle under investiga-
tion. To study muscle performance under physiological conditions, we recently developed a sensitive and accurate mouse isometric dynamometer (6). This approach enabled us to assess maximal tetanic torque and temporal parameters, such as rise time and relaxation time, of intact muscles in anesthetized mice.

METHODS

Animals. Mice lacking both mitochondrial and cytosolic CK (MM-CK−/− × MM-CK−/−, denoted as CK−/−) (15) were used in the present study. Male wild-type littermates (C57Bl/6 × 129/sv; 58–60 days old) served as controls. Halothane (Fluothane; Zeneca, Ridderkerk, The Netherlands) was used as an anesthesia agent supplied in O2 and N2O (3:1, 1.5–2.0%) via a facemask through a flowmeter system (4.0 l/min; Medec, Montvale, NJ). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Maastricht University and complied with the principles of laboratory animal care.

Surgical procedure. Mice were positioned on a thermoregulated platform (38.5 ± 0.1°C). A small incision in the lateral part of the knee was made to expose the peroneal nerve. After the skin was locally depilated, a bipolar platinum hook electrode was fixed to the skin with cyanoacrylate glue to prevent electrode displacement during muscle contraction. The electrode was changed, if a current of >1.0 mA was needed, to obtain maximal tetanic muscle contraction. The electrode was fixed to the skin with cyanoacrylate glue to prevent electrode displacement during muscle contraction.

Experimental measurement setup. For mechanical analysis, the anesthetized mouse was fixed via the hip and foot to the measurement device. Dorsal flexor torque around the ankle joint was measured with a sensitive and accurate custom-built device, the details of which were published earlier (6). Data acquisition was performed at 1,000 Hz with an Apple Macintosh 7100 PowerPC with an eight-channel, 12-bit Lab-NB analog-to-digital conversion board (National Instruments, Austin, TX). Postprocessing of the torque data was performed with Matlab 5.2.1 (The Math Works, Natick, MA).

Exercise protocol. Supramaximal stimulation current necessary to obtain recruitment of all muscle fibers was first determined using 3–5 isometric twitch contractions with increasing current. Resting periods between twitches were kept constant at 60 s.

Optimal muscle length at optimal ankle angle, was determined using 9 twitch contractions at ankle angles between 10° dorsal flexion and 30° plantar flexion. The ankle angle at which the muscle exerted maximal torque was not different between the wild-type and CK−/− mice and amounted to 19.1 ± 5.9 and 17.6 ± 4.0° of plantar flexion, respectively. Maximal tetanic torque was obtained with a 200-ms pulse train and 125-Hz stimulation frequency. Subsequently, dorsal flexors were subjected to 12 isometric tetanic contractions through electrical stimulation of the peroneal nerve for 170 ms every 265 ms (tour duty cycle: 0.64), at optimal ankle angle, 125 Hz, 0.5-ms pulse duration, and a supramaximal stimulation current.

Torque signals (n = 8) were digitized and analyzed for maximal tetanic torque, rise time, and half-relaxation time. Maximal torque was determined from filtered (11-point moving average) torque signals during electrical stimulation. Absolute tetanic torque values were normalized for the wet muscle complex mass (mN·m·g−1 muscle mass). Both the rise time and the half-relaxation time were determined from the unfiltered torque signals. The rise time is defined as the time required to increase the muscle torque from 10 to 50% of the maximal torque. The half-relaxation time is the time to decrease the torque from 50 to 25% of the maximal torque after cessation of electrical stimulation (5). Furthermore, the maximal torque value of each single contraction during the series of 12 contractions was determined. Temporal properties were characterized in terms of half-relaxation time of the 12th contraction and the rise time of the consecutive contractions.

Tissue sampling. Subsets of mice were used for muscular tissue sampling at two different time points during the contraction protocol for the analysis of tissue high-energy phosphates and related compounds, i.e., immediately after the series of 12 contractions (E; n = 6), and after 60 s of recovery (R) after E (E + R; n = 6). Control samples (C; n = 8) were taken from the resting contralateral dorsal flexor complex. Muscular tissue was rapidly freeze clamped using a pair of aluminum tongs cooled in liquid nitrogen. The tissue samples were stored at −80°C until analysis. For tissue sampling immediately after the contraction series (E), a slightly different experimental setup was used to prevent damage of the sensitive torque-measuring device. First, the optimal ankle angle and the supramaximal stimulation current were determined with the isometric mouse dynamometer. Thereafter, this device was replaced by a dummy apparatus, keeping the ankle angle and, hence, the muscle length at optimal value. The time interval between the final contraction of the series and the freezing procedure was kept as short as possible (<2 s).

Before high-energy phosphate analysis, the muscle samples were freeze dried overnight at −30°C. High-energy phosphates and related compounds were assessed with HPLC, as described by Wijnants and van Belle (22) and modified by van der Vusse et al. (18). Total adenine nucleotide content (TAN) was computed as the sum of ATP, ADP, and AMP. One set of contralateral dorsal flexor muscles from wild-type and CK−/− mice was used for determination of maximal activity of CK, adenylate kinase, and AMP deaminase. Briefly, muscle complexes were homogenized in SET buffer (0.25 M sucrose, 0.002 M EDTA, and 0.01 M Tris, at pH 7.4) and stored at −80°C. Maximal activity of CK (EC 2.7.3.2) and adenylate kinase (EC 2.7.4.3) was determined using the coupled-enzyme assay of hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), producing NADPH. AMP deaminase (EC 3.5.4.6) activity was assessed according to Rundell et al. (12). Maximal enzyme activities were measured with a centrifugal analyzer (Cobas Fara, La-Roche, Switzerland) and expressed as micromoles per minute per gram of muscular wet weight.

Number of animals and statistical procedures. In total, 14 CK−/− and 14 wild-type littermates were included in this study. Mechanical activity was measured in eight CK−/− and eight wild-type animals. The dorsal flexor muscle complex was freeze clamped 60 s after the high-intensity contraction series. Due to technical imperfections, the high-energy phosphate content was assessed in only six of eight muscle complexes. In a subset of six animals, the flexor muscle complex was freeze clamped immediately after the high-intensity contraction series and used for HPLC analysis (see Tissue sampling). The contralateral, resting flexor muscle complex was freeze clamped in 14 CK−/− and wild-type animals. Eight and five tissue samples were used for HPLC analysis and assessment of enzymatic activity, respectively. One sam-
were analyzed with Student’s t-test and during the contraction series using a one-way ANOVA. To analyze a possible interaction between the contraction conditions (C, E, and E + R) and the mouse type (wild-type vs. CK$^{-/-}$), a two-way ANOVA with Tukey’s post-hoc analysis was used to analyze the high-energy phosphate levels. The differences were considered significant if $P < 0.05$. SPSS 9.0 (SPSS Benelux, Gorinchem, The Netherlands) was used for statistical analyses.

**RESULTS**

Mean body mass of CK$^{-/-}$ mice (21.9 ± 1.7 g) was significantly lower than wild-type body mass (24.0 ± 1.0 g). Dorsal flexor mass of CK$^{-/-}$ mice amounted to 52.5 ± 6.1 mg and was significantly lower than that of the age-matched wild-type mice (62.1 ± 8.2 mg).

Figure 1 shows a representative maximal dorsal flexor torque pattern for wild-type and CK$^{-/-}$ mice at 125-Hz stimulation frequency. The mean values of isometric contractile parameters of dorsal flexors measured in CK$^{-/-}$ and wild-type mice are shown in Table 1.

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<tr>
<th>Table 1. Torque characteristics of a single tetanic contraction of dorsal flexor muscle of WT and CK$^{-/-}$ mice</th>
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<tbody>
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<td>WT</td>
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<tr>
<td>Tetanic torque, mN·m</td>
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<td>Mass normalized tetanic torque, mN·m·g wet wt$^{-1}$</td>
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<tr>
<td>Rise time, ms</td>
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<td>½-Relaxation time, ms</td>
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Values are means ± SD. Dorsal flexors ($n = 8$ in each group) were stimulated with 125 Hz, 0.5 ms, and a supramaximal stimulation current. Total duration of the tetanic contraction amounted to 170 ms. * Statistical differences between WT and CK$^{-/-}$, $P < 0.05$ and $P < 0.01$, respectively. WT, wild-type mice; CK$^{-/-}$, creatine kinase knockout mice.

Maximal tetanic torque at a stimulation frequency at 125 Hz was significantly higher in wild-type mice compared with CK$^{-/-}$ mice, which could be partially explained by the decline in muscle mass in CK$^{-/-}$ mice. However, after normalization of the values of the maximal tetanic torque on muscle mass, maximal tetanic torque was still significantly lower in CK$^{-/-}$ mice. The rate of relaxation after tetanic contraction was significantly slower in CK$^{-/-}$ than in control mice, whereas the rise time was not affected in CK$^{-/-}$ dorsal flexors (Table 1).

A representative torque output signal of a series of 12 consecutive tetanic contractions of dorsal flexors of wild-type and CK$^{-/-}$ mice is given in Fig. 2. In both cases, the maximal torque declined during the contraction series. Figure 2 also shows that the rates of torque buildup and relaxation are prolonged in CK$^{-/-}$ mice during the course of the 12 consecutive contractions.

The mean maximal torque of each individual contraction during the series of 12 tetanic contractions is depicted in Fig. 3. The maximal torque of both the wild-type and CK$^{-/-}$ mice showed an approximately linear decrease. The maximal torque of the 12th contraction was 12.0 ± 3.3% lower than that of the 1st contraction of wild-type mice. The maximal torque output of CK$^{-/-}$ mice tended to show a larger decrease...
at the last contraction (22.9 ± 9.8%), but due to the relatively high interindividual variation, this value was not significantly different from the corresponding value of wild-type mice.

The higher relaxation time, as observed in a single tetanic contraction, was even more conspicuous in the series of 12 tetanic contractions. The half-relaxation time at the 12th contraction of CK−/− mice was significantly longer compared with that of the wild-type mice, i.e., 26.2 ± 2.8 vs. 12.9 ± 3.5 ms. Furthermore, in CK−/− mice, the half-relaxation time of the 12th contraction was significantly longer than the half-relaxation time measured after a single tetanic contraction; this phenomenon was not present in experiments of wild-type mice. Moreover, the rise time of the first contraction was comparable to the values calculated during a single tetanic contraction, i.e., 11.1 ± 1.2 and 10.6 ± 1.2 ms for wild-type and CK−/− mice, respectively. Wild-type muscles showed no difference in rise time during the 12 consecutive contractions compared with the initial rise time in contrast to the CK−/−, which showed a substantial increase in rise time in the 2nd and 3rd contraction, i.e., 19.6 ± 3.0 and 29.4 ± 3.7 ms, respectively. The rise time of the 4th and succeeding contractions could not be calculated, because the relaxation was prolonged to such an extent that torque did not fall below 10% of the maximal torque value.

Table 2 shows the high-energy phosphate content of dorsal flexors at three different time points of the repeated contraction protocol for both wild-type and CK−/− mice.

Basal PCr and Cr levels were unaffected in CK−/− muscles, which resulted in comparable PCr-to-Cr ratios between CK−/− and wild-type muscles. Resting ATP values of CK−/− mice were significantly lower than those of wild-type mice, whereas the ADP and AMP content was significantly higher in CK−/− mice. The total of adenine nucleotides (TAN) was significantly lower in CK−/− dorsal flexors compared with wild-type mice. Furthermore, resting IMP levels showed a significant fourfold increase in CK−/− mice compared with wild-type control muscles.

Immediately after the series of 12 consecutive tetanic contractions, the PCr level in wild-type mice was significantly lower (ΔPCr = −16.7 μmol/g dry wt), whereas the Cr level increased to a comparable extent (ΔCr = 14.9 μmol/g dry wt). The PCr-to-Cr ratio of wild-type mice dropped from 1.6 ± 0.3 in resting conditions to 0.9 ± 0.2 after the contraction series.

In contrast, PCr and Cr levels in CK−/− dorsal flexors were not affected during the series of 12 tetanic contractions, which resulted in an unchanged PCr-to-Cr ratio. At the end of the contraction protocol, the CK−/− ATP level was significantly lower compared with the resting values. ADP and AMP levels did not change, whereas TAN significantly decreased. This contraction-induced decline in ATP content was absent in wild-type mice. Compared with resting values, CK−/− muscles showed a significant twofold increase in IMP content at the end of the series of tetanic contractions. In contrast, no accumulation of IMP occurred in wild-type muscles.

During the 60-s recovery period after the series of 12 tetanic contractions, PCr and Cr levels and the PCr-to-Cr ratio in wild-type mice returned to precontraction levels. In CK−/− muscles, recuperation of ATP levels did not occur during the recovery period, which was also the case for TAN. IMP levels in CK-mutant mice remained elevated during the 60-s recovery period.

The maximal activity of total CK in CK−/− muscle was on the order of 0.7% that of wild-type mice. No differences between wild-type and CK−/− dorsal flexors in maximal activity of adenylate kinase and AMP deaminase were found (Table 3).

**DISCUSSION**

Total CK activity in dorsal flexors of the double CK-knockout mice was reduced to ~0.7% that of wild-type animals. The residual activity can most likely be attributed to the CK-β isofrom present in vascular endothelium and muscle satellite cells. Using nuclear magnetic resonance analysis, Steeghs et al. (15) confirmed that PCr no longer could be used to regenerate

<table>
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<tr>
<th></th>
<th>WT (n = 8)</th>
<th>CK−/− (n = 8)</th>
<th>WT (n = 6)</th>
<th>CK−/− (n = 6)</th>
<th>WT (n = 6)</th>
<th>CK−/− (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>ATP</td>
<td>28.0 ± 2.1</td>
<td>20.1 ± 1.4</td>
<td>27.9 ± 2.0</td>
<td>15.5 ± 2.4</td>
<td>27.0 ± 3.4</td>
<td>14.4 ± 3.6</td>
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<td>ADP</td>
<td>4.0 ± 0.5</td>
<td>5.6 ± 1.2</td>
<td>3.7 ± 0.6</td>
<td>4.8 ± 1.3</td>
<td>3.8 ± 0.4</td>
<td>3.6 ± 1.0</td>
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<tr>
<td>AMP</td>
<td>0.2 ± 0.1</td>
<td>0.6 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.0</td>
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<tr>
<td>TAN</td>
<td>32.2 ± 2.3</td>
<td>26.3 ± 1.7</td>
<td>31.9 ± 2.5</td>
<td>20.7 ± 3.8</td>
<td>31.1 ± 3.4</td>
<td>18.3 ± 4.1</td>
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<td>IMP</td>
<td>0.3 ± 0.1</td>
<td>1.2 ± 0.5</td>
<td>0.2 ± 0.1</td>
<td>2.4 ± 1.1</td>
<td>0.2 ± 0.2</td>
<td>1.8 ± 1.1</td>
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<tr>
<td>PCr</td>
<td>73.7 ± 7.5</td>
<td>69.3 ± 6.6</td>
<td>57.0 ± 4.7</td>
<td>65.0 ± 2.1</td>
<td>80.3 ± 7.8</td>
<td>66.1 ± 9.0</td>
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<tr>
<td>Cr</td>
<td>46.3 ± 5.8</td>
<td>42.6 ± 5.2</td>
<td>61.2 ± 12.6</td>
<td>37.2 ± 2.8</td>
<td>39.1 ± 3.5</td>
<td>39.6 ± 3.5</td>
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<tr>
<td>PCr + Cr</td>
<td>120.0 ± 8.3</td>
<td>119.1 ± 11.4</td>
<td>116.2 ± 13.8</td>
<td>102.2 ± 4.8</td>
<td>119.3 ± 7.9</td>
<td>105.7 ± 12.1</td>
</tr>
<tr>
<td>PCr/Cr</td>
<td>1.6 ± 0.3</td>
<td>1.6 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>2.1 ± 0.3</td>
<td>1.7 ± 0.2</td>
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Values are means ± SD, expressed as micromoles per gram dry weight. E, resting values; E, content measured in tissue freeze-clamped immediately after a series of 12 tetanic contractions; E + R, content measured in tissue freeze-clamped at the end of a 60-s recovery period after a series of 12 tetanic contractions; TAN, total adenine nucleotide content. The ratio phosphocreatine (PCr)/creatinine (Cr) is dimensionless. *Statistical differences between WT and CK−/− mice in resting conditions, P < 0.05 and P < 0.01, respectively; ‡statistical differences between either E or E + R with C, P < 0.05.
hydrolyzed ATP in CK−/− mice. Hence, double-CK-deficient mice are a useful model to study the impact of a lack of CK on muscle contractile properties and adenine nucleotide handling during high-intensity contractions.

Single isometric tetanic contraction. The present study clearly shows that the maximal tetanic torque output of CK−/− mice is significantly declined. This finding implies that the lack of CK activity substantially affects contractile function of intact muscles investigated in their natural environment. Our in situ observation corroborates earlier findings obtained with isolated muscles, showing a decline in the force of muscles lacking CK activity (4, 14, 16). Theoretically, alterations in the maximal value of muscle torque can be caused, among other factors, by variation in muscle mass, altered length of the muscle lever arm, and/or changes in energy metabolism. We observed a 16% decline in dorsal flexor muscular mass of the CK−/− mouse. After normalization of maximal tetanic torque on muscle mass, torque per gram of tissue was still 29% lower in CK−/− mice than that in age-matched controls. It is of interest to note that Steeghs et al. (14) previously observed a 30% decline in maximal force output in isolated gastrocnemius medialis muscle of CK−/− mice. These combined findings strongly suggest that alterations in lever arm do not likely occur in CK-mutant mice, indicating that additional factors are involved in the decline of maximal torque.

Altered levels of high-energy phosphates and related compounds in skeletal muscle of CK−/− mice may play a role in the decrease in maximal torque. In the past, a variety of substances has been put forward as likely candidates that can negatively affect muscular contractility, such as raised concentrations of Pi and IMP or low ATP levels (1, 20). Differences in tissue pH should be also taken into consideration.

A decisive role of hydrogen ions in depressed muscular function of CK−/− mice is less likely. First, Steeghs et al. (14) recently showed that pH in both resting and contracting CK−/− muscles did not differ from wild-type muscles. Second, the production of lactate during contractions of EDL and soleus muscles was even smaller in CK−/− mice than in corresponding muscles of wild-type littermates (4). It is of interest to note that Dahlstedt et al. (4) recently reported a twofold increase in resting inorganic phosphate (Pi) levels in EDL muscle of CK−/− mice. Because Pi is known to affect the strong cross-bridge attachment and myofibrillar Ca2+ sensitivity (9), higher cellular Pi concentrations may be a causative factor in the decline of maximal torque of the CK−/− muscles. Furthermore, the resting IMP level in the dorsal flexor muscle was found to be increased fourfold in CK−/− mice (Table 2), which indicates a change in the ATP-regenerating process in muscle cells lacking CK activity (13). Although the precise mechanisms of action are unclear (7), the results obtained in vitro studies suggest that IMP may interfere with Mg2+-myosin ATPase activity (21), hampering the formation of the myosin-actin complex. In this light, the substantially elevated IMP content in the dorsal flexor muscle of CK−/− mice should be considered a likely candidate for involvement in the decline in torque output. The significantly lower ATP content in resting muscle of the CK−/− mouse may also contribute to the lower torque output. Westerblad et al. (20) observed a correlation between reduced muscular ATP levels and a rise in intracellular Ca2+ content, suggesting a possible role of ATP via calcium ions in impaired muscular function of CK−/− mice.

However, Dahlstedt et al. (4) recently showed that, with the use of a single fiber preparation, tetanic Ca2+ levels did not differ between CK−/− and control mice. This finding dismisses the possibility that a low cellular ATP level negatively influences torque output via alterations in Ca2+ handling. An alternative mechanism might be a direct effect of lower ATP levels on myosin ATPase activity and, hence, on the maximal tetanic torque output via reduced cross-bridge cycling.

The half-relaxation time of a single maximal tetanic contraction was significantly higher (~42%) in CK−/− mice than in wild-type mice. Our findings in intact animals are in line with previous observations that the relaxation time of isolated muscles of CK−/− mice is prolonged (14). The combined results point toward a disturbed reuptake of Ca2+ after muscle contraction, in which process energy-dependent Ca2+-ATPase pumps (SERCA2) in the sarcoplasmic reticulum (SR) are the key players (11). It should be emphasized, however, that no differences in mRNA and protein content or activity of SERCA2 were found between CK−/− and wild-type gastrocnemius muscle (14). Therefore, if Ca2+ reuptake in the SR is impaired in CK−/− muscles, this process may be directly affected by alterations in the energy status of the muscle cell, i.e., no temporal buffering of ATP by PCr and, hence, a decline in ATP concentration at the site of action.

Repetitive tetanic contractions. When dorsal flexor muscles of CK−/− and wild-type mice were energetically challenged by a series of 12 high-intensity tetanic contractions, tour duty cycle 0.64, several interesting differences in torque output became apparent. Although the decline in maximal torque during the course of 12 tetanic contractions tended to be greater in CK−/− muscles, the difference between CK−/− and wild-type muscles did not reach the level of significance. The relaxation time significantly increased in CK−/− mice during the series of 12 contractions.

Table 3. Maximal activity of total CK, adenylate kinase and AMP deaminase in dorsal flexor muscles of WT and CK−/− mice

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<thead>
<tr>
<th></th>
<th>WT</th>
<th>CK−/−</th>
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<tr>
<td>Total creatine kinase</td>
<td>5.668 ± 832</td>
<td>37 ± 10†</td>
</tr>
<tr>
<td>Adenylate kinase</td>
<td>36.9 ± 7.9</td>
<td>30.7 ± 3.9</td>
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<tr>
<td>AMP deaminase</td>
<td>51.6 ± 14.5</td>
<td>48.4 ± 12.7</td>
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Values are means ± SD (n = 5 for each group) in units per gram wet weight. †Statistical differences between WT and CK−/−, P < 0.01.
In wild-type muscles, the relaxation time remained unchanged. The rise time became significantly longer in CK−/− muscles, whereas the rise time in wild-type muscles remained constant during the consecutive contractions. These phenomena indicate impaired tolerance of muscles, lacking both mitochondrial and cytoplasmic CK, toward high-intensity contractile activity. Because the present experimental approach does not allow elucidation of the precise mechanisms underlying the increased intolerance, one may speculate that the lack of buffering of ATP by PCr is attributable to a great extent to the accelerated decline in rate of developed torque and lengthening of relaxation time.

Assuming that in wild-type muscle the major mechanism of ATP regeneration during the series of 12 high-intensity contractions is rephosphorylation of ADP by PCr, an amount on the order of 16.7 μmol ∼ P/g dry wt is consumed during the course of the contractile activity. This value is calculated on the basis of the mean of the absolute decline in PCr and the absolute increase in Cr (Table 2). During the 12 consecutive contractions, the total torque time integral amounted to 85.7 ± 12.1 and 58.9 ± 12.5 mN · m · s · g⁻¹ for wild-type and CK−/− dorsal flexors, respectively. Assuming that the torque time integral normalized per unit weight of tissue linearly relates to chemical energy consumed, it can be estimated that the amount of ~P utilized in CK−/− dorsal flexor muscles during the series of 12 contractions will be 69% (11.5 μmol/g dry wt) of that of wild-type muscles (16.7 μmol/g dry wt). As expected, the tissue content of PCr did not change, underscoring the fact that, due to the lack of CK, PCr cannot be used to buffer the ATP hydrolyzed during mechanical activity of the muscle fiber (14). The concomitant increase in IMP content indicates that (part of) ADP is rephosphorylated by the catalytic action of adenylate kinase (Fig. 4).

Because for each ATP molecule regenerated via the adenylate kinase pathway one molecule of AMP is formed, the decline of 4.6 μmol ATP/g dry wt indicates that ≥9.2 μmol ATP/g dry wt are hydrolyzed during the series of high-intensity contractions in the CK−/− muscle, neglecting the contribution of the anaerobic glycolysis. This value is in close accord with the amount of ATP utilized as estimated from the developed torque, i.e., 11.5 μmol/g dry wt. It should be emphasized that, in CK−/− flexor muscles, AMP was not significantly elevated at the end of the contraction series, indicating that, once formed, AMP is immediately deaminated by action of AMP deaminase. The present findings also show that accumulation of IMP is less than the decline in ATP content, i.e., 1.3 vs. 4.6 μmol/g dry wt. This difference strongly suggests that IMP is further degraded to compounds such as inosine, hypoxanthine, and xanthine. Chemical analysis of the dorsal flexor muscle tissue at the end of the series of high-intensity contractions did not, however, show an increase in the tissue content of IMP degradation products. This can be explained by the fact that muscular membranes are not barriers for (oxy)purines, allowing the release of these low molecular weight compounds from the contracting muscle into the vascular compartment. The rapid conversion of AMP into IMP and further degradation to (oxy)purines during contractile activity may also explain the substantially lower ATP content in resting muscle of CK−/− compared with wild-type mice, because the de novo synthesis of ATP via the purine salvage pathway is a relatively slow process (10). This notion is underscored by the present observation that, in muscle tissue of CK−/− mice, TAN did not recover during 60 s of rest after the series of tetanic contractions.

The maximal activity of adenylate kinase and AMP deaminase, required for the formation of AMP and its subsequent degradation to IMP, was not affected in muscles of CK−/− mice (Table 3). In vitro studies have shown that the activity of AMP deaminase in CK−/− is pH dependent (10). Because Steeghs et al. (14) reported that changes in pH during mechanical activity of muscles deficient in CK did not differ from wild-type muscles, the potential regulatory role of H⁺ can be dismissed under our experimental conditions. Moreover, it is of interest to note that Tullson et al. (17) obtained evidence that the affinity of AMP deaminase for AMP is appreciably enhanced in CK−/− muscles, which may explain the higher rate of IMP formation in the absence of a measurable increase in the tissue content of AMP.

In summary, our results show that the torque output of the in situ dorsal flexor complex of CK−/− mice is significantly depressed. This decline can be only partially explained by the decrease in muscle mass. Lower tissue ATP and higher IMP levels may contribute to the decline in torque during a high-intensity tetanic.

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**Fig. 4.** Schematic representation of the phosphate flux through the adenylate kinase pathway and subsequent reactions in dorsal flexor muscle of CK−/− mice during a series of 12 tetanic contractions. Values are expressed as micromoles per gram dry weight per contraction series. NS, no significant increase (+) or decline (−) in tissue content; a, b, and c refer to adenylate kinase, AMP deaminase, and 5'-nucleotidase, respectively. Note that, via the adenylate kinase pathway, for each molecule of ATP regenerated, 1 molecule of AMP is produced and, via AMP deaminase, is degraded to IMP and subsequently to (oxy)purines. This process results in a decline of the adenine nucleotide pool.
contraction. In contrast to wild-type muscle, ATP content could not be maintained during a series of 12 tetanic contractions in dorsal flexor muscle of CK−/− mice. The increase in tissue IMP indicates that adenylate kinase-mediated regeneration of ATP compensates for the lack of CK−/− activity. However, this pathway inevitably results in a decline in tissue content of ATP.

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