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Physiology

Creatine kinase (CK) in skeletal muscle energy metabolism: A study of mouse mutants with graded reduction in muscle CK expression

(phosphocreatine system/muscle bioenergetics/homologous recombination/embryonic stem cells)

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ABSTRACT **To understand better the role of the creatine kinase (CK)/phosphocreatine system in muscle bioenergetics, a series of mouse mutants with subnormal muscle CK (M-CK) expression has been generated. Here we compare the phenotypes of mice deficient in M-CK (M-CK$^{-/-}$) and M-CK leaky-mutant mice, which carry a targeted insertion of a hygromycin B–poly(A) resistance cassette in the second M-CK intron. Mice homozygous for this M-CK allele (M-CK$^{I/I}$) have a 3-fold reduction of dimeric muscle CK enzyme activity, whereas compound heterozygotes with the null M-CK allele (M-CK$^{I/-}$) display a 6-fold reduction. Unlike M-CK$^{-/-}$ mice, these mutants have no increased glycogen content or glycogen consumption in their fast fibers. The intermyofibrillar mitochondrial volume of these fibers is also normal, suggesting that energy transport via the CK/phosphocreatine system may function at low myofibrillar M-band CK levels. Conversely, the flux of energy through the CK reaction is still not visible by means of ^{31}P NMR spectroscopy, indicating that relatively high levels of M-CK expression (>34% of normal) are required to generate CK fluxes detectable by this technique. The ability of muscles to perform burst activity is also subnormal and closely correlates with the level of M-CK expression.**

Creatine kinase (CK; EC 2.7.3.2) isoenzymes are abundantly present in cells and tissues with high and fluctuating energy demands, where they catalyze the exchange of high-energy phosphates between phosphocreatine (PCr) and ATP via the reaction: PCr + ADP + H^+ ⇄ Cr + ATP (1, 2). Substrates and enzymes involved in the CK reaction together form the so-called CK/PCr system, and its role in energy metabolism has been extensively studied in skeletal muscle. The cellular PCr pool is primarily considered to function as an energy store that buffers changes in ATP levels during periods of muscle exercise by replenishing hydrolyzed ATP via the CK reaction catalyzed by the dimeric muscle CK enzyme (MM-CK) (3, 4). In addition, the CK/PCr system is presumed to play a key role in the transport of energy between subcellular sites of ATP production and consumption, in which PCr and Cr act as the shuttle molecules (1, 5).

We have previously described a knock-out mutation of the muscle CK (M-CK) gene that was introduced into the germ line of mice (6). Mice deficient in M-CK are viable and exhibit no overt abnormalities. Though their maximal muscle force is normal, they lack the ability to sustain maximal muscle output during short periods of high-resistance work. Interestingly, mutant muscles appear to develop better endurance performance. Fast-twitch (type 2A and 2B) fibers selectively exhibit an increased intermyofibrillar mitochondrial volume and an increased glycogenolytic/glycolytic potential. PCr and ATP levels and the ability to hydrolyze PCr during contraction are normal in muscles without MM-CK, although the energy flux through the CK reaction is at least 20-fold below normal. These findings demonstrate a crucial physiological role of the CK/PCr system in burst performance and show the high plasticity of cellular architecture and energy metabolism of muscle tissue.

The complexity of the phenotypic alterations in mice without M-CK is such that the direct consequences of gene inactivation may be obscured by possible secondary effects. M-CK functions may be better resolved if effects of a series of mutations are evaluated. Here we report a mutation in the M-CK gene (M-CK^I allele) that resulted from an unpredicted insertion of a hygromycin B (hygroB)–poly(A) resistance cassette in the second M-CK intron and caused a reduction in muscle MM-CK activity of ≈3-fold. By crossing M-CK$^{I/I}$ wild-type, and knock-out mice, offspring were generated with graded levels in MM-CK activity, and their phenotypic alterations were compared.

MATERIALS AND METHODS

Generation of M-CK$^{I/I}$ Mice. Generation and injection of AB-1-822 embryonic stem cells into C57BL/6 blastocysts were as described (6, 7). Male chimeras were mated with C57BL/6 females. M-CK$^{+/I}$ heterozygotes, identified by Southern blot analysis, were paired to obtain homozygous mutant mice.

Southern and Northern Analysis. Genomic DNAs were isolated from tail biopsies or spleens, and Southern blot analyses were performed as described (8). Blots were subsequently probed with 3′-M-CK (7), M-CK-ATG (6), 5′-M-CK (6), and Hygro (7). Total RNA was isolated from skeletal muscle and heart (9). Northern blots were prepared and subsequently probed with M-CK-ATG and glyceraldehyde-3-phosphate dehydrogenase DNA as reported (6).

Histochemistry. Cross sections of gastrocnemius–plantaris–soleus (GPS) muscle groups were stained for succinate dehydrogenase (SDH) activity as described in ref. 6.

Enzyme Activity and Energy Metabolite Analyses. Skeletal muscle extracts for analysis of enzyme activities were prepared as described in detail (6). Total CK activity was measured at 30°C on a COBAS-Mira analyzer (Hoffmann–La Roche) using the CK activity kit (no. 475742, Boehringer Mannheim) (10). Cytochrome *c* oxidase and citrate synthase were measured as described (6).

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Abbreviations: CK, creatine kinase; PCr, phosphocreatine; M-CK, muscle CK; MM-CK, dimeric muscle CK enzyme; Mi-CK, mitochondrial CK; GPS, gastrocnemius–plantaris–soleus; hygroB, hygromycin B; SDH, succinate dehydrogenase.

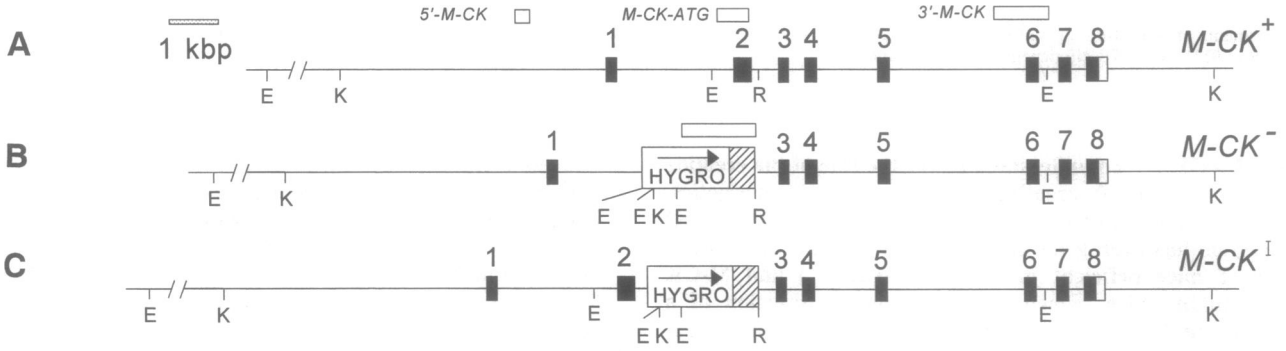
ATP and glycogen (before and after muscle exercise) were quantitated by standard enzymatic analyses as described (6).

NMR Spectroscopy. ^{31}P NMR PCr inversion transfer experiments on upper hind-limb muscles of M-CK$^{+/-}$, M-CK$^{I/I}$, and M-CK$^{I/-}$ mice at rest (n = 4 in each group) were performed as described in detail in ref. 6. At the beginning of each experiment, a fully relaxed spectrum was collected to determine the relative peak areas of γ-ATP, α-ATP, β-ATP, PCr, and P_i.

Muscle Force Measurements. Muscle force measurements were carried out as described (6). We determined successively the maximal twitch force of three single contractions and the twitch force of the first 24 contractions at 5-Hz electrostimulation.

RESULTS

Generation of the M-CK^{I} Allele and M-CK$^{I/I}$ Mice. In a previous report (7), we described the targeted inactivation of the M-CK gene in AB-1 embryonic stem cells using an isogenic vector (129-pRV8.3), which was designed to replace part of introns 1 and 2 and exon 2 (0.9 kbp) by a hygroB–



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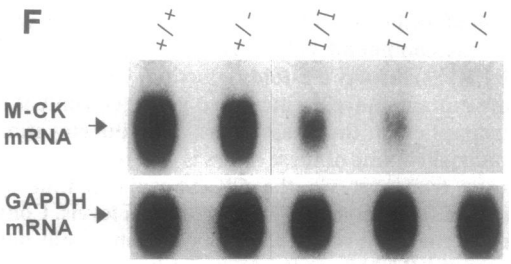
PROBES	5'-M-CK		3'-M-CK		M-CK-ATG		HYGRO	
ENZYME(S)	K	E/R	K	E/R	K	E/R	K	E/R
WILD-TYPE	18.0	15.0	18.0	5.6	18.0	0.9	-	-
KNOCK-OUT	8.0	15.0	10.7	5.6	-	-	10.7	1.4
Intron 2-insertion	9.1	15.0	10.7	5.6	9.1	1.05	10.7	1.4



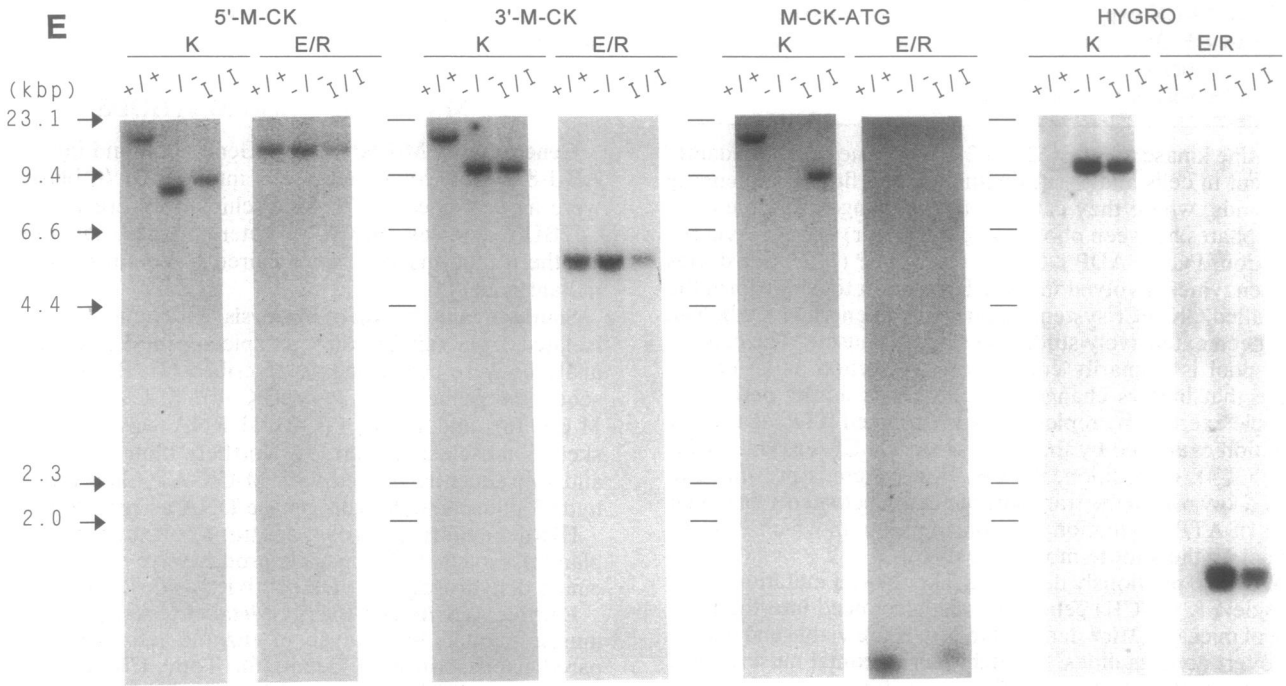


FIG. 1. Mutations in the M-CK gene introduced by gene targeting and their influence on M-CK mRNA levels in skeletal muscle. Schematic diagrams showing the genomic structures of wild-type M-CK (*A*), inactivated M-CK (*B*), and M-CK with an insertion of the hygroB–poly(A) cassette in the second M-CK intron (*C*). Numbers above black boxes indicate exons 1–8, and open bars denote the positions of the 5′-M-CK, M-CK-ATG, 3′-M-CK, and hygroB probes. (*D*) Table of approximated fragment sizes (kbp) hybridizing to the four probes indicated above. (*E*) Southern blot analyses of DNA from controls (+/+), mice homozygous for the inactivated M-CK allele (−/−), and mice homozygous for the M-CK^{I} allele (I/I). Positions of DNA marker fragments are indicated at the left site. E, *Eco*RI; K, *Kpn* I; R, *Eco*RV. (*F*) Northern blot analysis of total RNA (5 μg) prepared from upper hind-limb muscles and hybridized with an M-CK exon 2 and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (for RNA quantitation). Mouse genotypes are indicated above the lanes (+/−, heterozygote carrying a wild-type and an inactivated M-CK allele; I/−, heterozygote with a M-CK^{I} allele and an inactivated M-CK allele).

poly(A) resistance cassette (2.0 kbp). On Southern blots with *Kpn* I-digested DNAs, a total of 25 independent targeted clones were identified that yielded the wild-type 18.0-kbp and the mutant 10.7-kbp diagnostic fragments with a 3′ external M-CK probe (Fig. 1). However, when an exon 2 probe (M-CK-ATG) was used to verify further the integrity of the homologous recombinations, we observed that one of the targeted clones (AB-1-822) carried an unexpected 9.1-kbp *Kpn* I fragment, suggesting that exon 2 sequences were still present in the targeted allele of this clone (Fig. 1*E*, M-CK-ATG gels, lane I/I). Subsequent analysis with two additional probes, a 5′ external probe (5′-M-CK) and a hygroB probe, and additional digestions with *Eco*RI/*Eco*RV (Fig. 1*E*) and *Eco*RI (data not shown) revealed that the hygroB–poly(A) resistance cassette had inserted into M-CK intron 2 without replacing the 0.9-kbp gene fragment containing exon 2. The presumed structure of the incorrectly targeted M-CK allele in clone AB-1-822, referred to as M-CK^{I}, is illustrated in Fig. 1*C*.

AB-1-822 embryonic stem cells were injected into 3.5 days postcoitum C57BL/6 blastocysts, and chimeric mice transmitting the M-CK^{I} allele to their offspring were obtained. Heterozygous mutants, when interbred, yielded progeny homozygous for the M-CK^{I} allele. M-CK$^{I/I}$ mutants were viable and displayed no overt abnormal phenotype. Southern blot analyses of tail DNAs from M-CK$^{I/I}$, M-CK$^{-/-}$, and M-CK$^{+/+}$ mice are shown in Fig. 1*E*.

M-CK-mRNA Levels and MM-CK Activity in Muscles of M-CK$^{I/I}$ and M-CK$^{I/-}$ Mice. In theory, the M-CK^{I} allele can encode two kinds of mRNA if transcription initiation is mediated via the M-CK promoter region. A M-CK-hygroB fusion messenger (≈2600 nt) may be derived from a primary transcript if the first polyadenylylation site, the hygroB–poly(A) site located in the second M-CK intron, is used. An otherwise fully normal 1415-nt M-CK mRNA (11) may be produced if transcription proceeds beyond this site and the native M-CK polyadenylylation signal is used, provided that the hygroB gene sequences are correctly excised from the primary transcript by splicing. Northern blots of RNA isolated from skeletal muscles of M-CK$^{I/I}$ mice hybridized with a M-CK exon 2 probe showed expression of the normal 1415-nt M-CK mRNA (Fig. 1*F*), although, at a much reduced intensity. The anticipated M-CK–hygroB fusion mRNAs were undetectable, indicating that such transcripts were either not formed or were not stable. We sought to determine whether the expression levels of M-CK could be reduced further by crossing M-CK$^{I/I}$ and M-CK$^{-/-}$ mice. As expected, M-CK mRNA levels in skeletal muscles of M-CK$^{I/-}$ mice were found to be reduced below those of M-CK$^{I/I}$ mice (Fig. 1*F*).

Zymogram assays (available on request) showed that the graded reduction of M-CK mRNA levels in M-CK$^{I/I}$ and M-CK$^{I/-}$ skeletal muscles is accompanied by a stepwise decrease of MM-CK activity. Activities of the other CK isoforms were normal in both mutant mice, suggesting that the decrease in MM-CK activity did not affect the levels of other members of the CK family; even the ≈2-fold increase of mitochondrial CK (Mi-CK) activity observed in skeletal muscles of null mutants (6) was not noticed. The reductions of skeletal muscle MM-CK activity were quantitated by measuring the total CK activities (Table 1) with a standardized CK activity assay (10). In all genotypes analyzed, the total M-CK activity represents the sum of MM-CK and Mi-CK activities (traces of brain CK activity are negligible) except for M-CK$^{-/-}$ mice, where the total CK activity is equal to the Mi-CK activity. If we assume that muscle Mi-CK activities of M-CK$^{+/+}$, M-CK$^{+/-}$, M-CK$^{I/I}$, and M-CK$^{I/-}$ mice are half of those of M-CK$^{-/-}$ mice (6), then MM-CK activity in the various genotypes can be simply derived from the total CK activity (Table 1). Compared to controls, muscle MM-CK activity is reduced approximately 2-, 3-, and 6-fold in M-CK$^{+/-}$, M-CK$^{I/I}$, and M-CK$^{I/-}$ mice, respectively.

Table 1. Activities of CKs and mitochondrial enzymes in upper hind-limb muscles of M-CK mutant mice

Genotype	CK_{tot}	MM-CK	COX	CS
+/+	41,634 ± 7644	41,286 ± 7644	604 ± 28	127 ± 32
+/−	21,698 ± 1255	21,350 ± 1257	551 ± 96	148 ± 23
I/I	14,364 ± 2263	14,016 ± 2264	730 ± 118	153 ± 12
I/−	7,129 ± 902	6,781 ± 906	776 ± 112	181 ± 20
−/−	695 ± 162	0	1094 ± 230	231 ± 27

Values are means ± SD and expressed in milliunits/mg of protein; $n = 8$ for +/+ and −/−; $n = 6$ for +/−, I/I, and I/−. All enzyme activities of M-CK mutant mice are significantly different from controls ($P < 0.05$), except for the cytochrome *c* oxidase and citrate synthase values of +/− and I/I mice. CK_{tot}, total CK activity; COX, cytochrome *c* oxidase; CS, citrate synthase.

Normal Mitochondrial Distribution and Glycogen Content in Fast Fibers of M-CK$^{I/I}$ and M-CK$^{I/-}$ Mice. To compare phenotypic alterations in type 2A and 2B fibers of mice with reduced MM-CK activities to those seen in M-CK-deficient mice (6), cross sections of M-CK$^{I/I}$ and M-CK$^{I/-}$ GPS muscle complexes were stained for SDH activity. Both genotypes displayed a similar SDH staining pattern as type 2 fibers of controls, and the coarsely granulated aspect, which is indicative for an increased intermyofibrillar mitochondrial volume in null mutant mice, was not observed (Fig. 2). Transmission electron microscopy confirmed that type 2 fibers of mice with 3- and 6-fold reduced MM-CK had a normal appearance (data not shown). Moreover, also no obvious increase in the number of enlarged mitochondria was observed.

The activities of two mitochondrial enzymes, cytochrome *c* oxidase and citrate synthase, as markers for the aerobic potential, were in keeping with these observations (Table 2). Activities in M-CK$^{I/I}$ and M-CK$^{I/-}$ muscles were found moderately elevated (on average 17% and 25% increase, respectively) compared to values in M-CK$^{+/+}$ and M-CK$^{+/-}$ muscles. However, only activities of M-CK$^{I/-}$ muscles are sufficiently different from controls to be significant ($P < 0.05$). It is conceivable that this adaptation in aerobic energy-

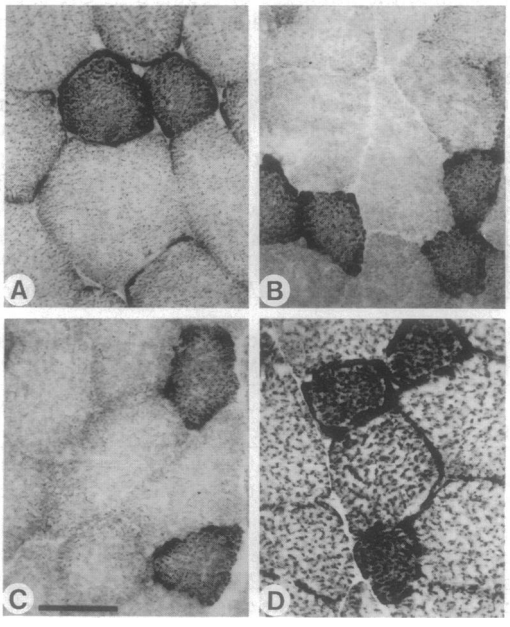


FIG. 2. Cross sections of gastrocnemius muscles with different levels of M-CK expression stained for SDH activity. (*A*) M-CK$^{+/+}$. (*B*) M-CK$^{I/I}$. (*C*) M-CK$^{I/-}$. (*D*) M-CK$^{-/-}$. Type 2A fibers are intensely stained, whereas type 2B fibers are lightly stained. (Bar = 30 μm.)

Table 2. Energy metabolites in skeletal muscles of mice with reduced M-CK expression

Metabolite	Genotype				
	+/+	+/−	I/I	I/−	−/−
P (NMR)	5.8 ± 1.7	4.9 ± 0.5	4.6 ± 1.3	4.6 ± 0.8	5.1 ± 0.9
PCr (NMR)	44.9 ± 2.9	47.0 ± 1.6	46.7 ± 0.6	44.2 ± 1.2	46.6 ± 2.2
ATP (NMR)	16.4 ± 1.6	16.0 ± 2.3	16.4 ± 1.6	17.0 ± 2.1	16.1 ± 1.4
ATP (chem)	7.9 ± 0.5	7.7 ± 0.7	7.0 ± 0.4	7.0 ± 0.6	7.0 ± 0.6
− glycogen	26.1 ± 5.8	ND	18.0 ± 7.6	19.7 ± 5.4	42.3 ± 7.2*
+ glycogen	17.3 ± 4.1	ND	12.7 ± 3.8	14.0 ± 4.0	25.3 ± 3.5*

NMR values are given as percentages and relate the metabolite peak area to the sum of the peak areas (the ATP values are means of the α-, β-, and γ-ATP peak areas) (n = 6). ATP (chem), absolute ATP concentration determined chemically (given in μmol/g of wet weight); + glycogen, glycogen content determined after 3 min of muscle stimulation at 5 Hz (given in μmol of glucose per gram of wet weight); − glycogen, glycogen content determined in nonstimulated muscle. ATP (chem) and glycogen values are means ± SD; n = 6 for +/+ and −/−, n = 5 for I/I, and n = 4 for +/− and I/−. ND, Not determined.

*Values significantly different ($P < 0.05$) from those of wild-type muscle.

generating capacity involves a small enlargement of mitochondrial size and/or mitochondrial reticulum that is undetectable by electron microscopy (12).

Chemical quantification of the glycogen levels in GPS muscle complexes of the two mutants with partial MM-CK activity revealed values somewhat below those of controls, although these differences were again not statistically significant. In addition, the rate of glycogen breakdown was also similar to control values (Table 2). Thus, the aerobic as well as the anaerobic energy potential seems to be normal in skeletal muscles with a 3- or 6-fold decline in MM-CK activity.

No NMR Detectable CK Flux in Muscles of M-CK$^{I/I}$ and M-CK$^{I/-}$ Mice at Rest. As in null mutants (6), the ATP concentration determined chemically was somewhat lower in GPS muscles of M-CK$^{I/I}$ and M-CK$^{I/-}$ mice than in wild-type muscles (Table 2), but differences were not significant. ^{31}P NMR spectra (available on request) of intact hind-limb muscles of anesthetized mice revealed that relative peak areas of ATP, PCr, and inorganic phosphate (P_i) resonances were similar in all genotypes, indicating that the levels of these metabolites are independent from the MM-CK content.

To determine the influence of reduced M-CK expression on the NMR detectable high-energy phosphate flux through the muscle CK reaction, we used the ^{31}P NMR inversion transfer technique (13). Measurements of energy fluxes in hind-limb muscles in the direction from ATP to PCr (PCr inversion) are listed in Table 3. Surprisingly, as in M-CK-deficient muscles (6), the degree of high-energy phosphoryl transfer in muscles with 3- and 6-fold-reduced MM-CK activity was found to be below the level of ^{31}P NMR detection. Based on considerations about signal-to-noise limitations (13), we estimate that the CK flux in these muscles is at least 20-fold below normal. The CK flux in muscles of M-CK$^{+/-}$ mice was somewhat lower than in those of controls, but differences were insignificant ($P < 0.05$).

Table 3. Kinetics of the CK reaction in intact upper hind limb muscles from mice with reduced M-CK expression

Genotype	MM-CK activity, %	K_r, s^{-1}	Reverse flux, μmol·g^{-1}·s^{-1}
+/+	100	0.95 ± 0.12	7.51 ± 1.06
+/−	50	0.79 ± 0.05	6.08 ± 0.67
I/I	34	≤0.05	≤0.38
I/−	16	≤0.05	≤0.38
−/−	0	≤0.05	≤0.38

Reverse pseudo-first-order rate constants (K_r) for the high-energy phosphate transfer from ATP to PCr were derived from NMR inversion transfer studies (PCr inversion) as described (6). Reverse CK fluxes were calculated from the reverse rate constants and the ATP concentrations (Table 2). The K_r values and reverse fluxes of I/I, I/−, and −/− mice are given as 5% of +/+ values. Values are means ± SD; n = 4 for all genotypes.

M-CK$^{I/I}$ and M-CK$^{I/-}$ Muscles Have Reduced Burst Activity. We monitored the development of force in GPS complexes at the onset of stimulation. Within the first eight twitches at 5-Hz stimulation, muscle force dropped about 45%, 13%, 9%, and 4% in M-CK$^{-/-}$, M-CK$^{I/-}$, M-CK$^{I/I}$, and M-CK$^{+/+}$ muscles, respectively, whereas in controls muscle force remained maximal (Fig. 3). Thereafter, muscle force remained virtually constant in all different genotypes, except for null mutants in which muscle force recovered slightly. The maximal twitch force of muscles was identical for all genotypes.

DISCUSSION

Graded Reductions in M-CK Expression. Levels of M-CK mRNA in skeletal muscle of mice homozygous for the newly identified M-CK^{I} allele were found to be markedly reduced. Most likely, the majority of primary transcripts initiating at the M-CK promoter are polyadenylylated at the poly(A) site of the hygroB cassette in the second M-CK intron of this allele and, as a consequence, are lost from the pool of mRNAs. It is conceivable that reduced processing efficiency or reduced stability of the primary transcripts are involved in this phenomenon, but also transcription distortion or other effects cannot be excluded.

In wild-type mice, both M-CK alleles contribute to the production of mRNA and M-CK subunits, because mRNA and MM-CK activity levels declined in heterozygotes with only a single functional M-CK allele. Similarly, M-CK expression in M-CK$^{I/I}$ mice was about 2-fold higher than in mice with one M-CK^{I} allele. These data indicate that the level of MM-CK dimers in skeletal muscle is strictly determined by gene dose and transcriptional activity.

Phenotypic Comparison of a Set of M-CK Mutants with Reduced MM-CK Activity. A very conspicuous adaptation in type 2 fibers of null mutants (6) results in a cellular architecture with an elaborated intermyofibrillar mitochondrial network. This may function to facilitate energy transport directly via ATP and ADP shuttle molecules, of which ADP has a small diffusion radius (14). If this explanation holds true, then the observation of normal intermyofibrillar mitochondrial volume in M-CK$^{I/I}$ and M-CK$^{I/-}$ mice reported here suggests that energy transport using PCr and Cr as shuttle molecules (1, 5) still functions at levels of MM-CK activity 3–6 times below normal. As glycogen content and consumption were also not noticeably altered in these mutants, we surmise that changes in anaerobic energy metabolism and cellular architecture, as seen in null mutants, may be closely linked adaptations.

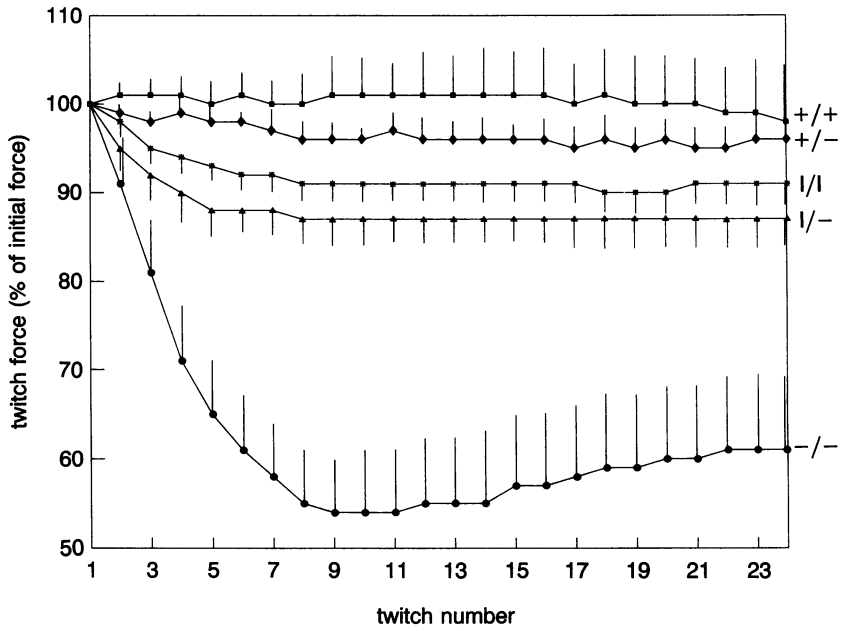


FIG. 3. Muscle performance of mice with reduced M-CK expression. The isometric twitch force during the first 24 contractions of GPS muscle complex at 5-Hz electrostimulation was recorded. Shown are means plus or minus the SD of 7 muscles. +/+, wild-type muscle force; +/−, M-CK$^{+/-}$ force; I/I, M-CK$^{I/I}$ force; I/−, M-CK$^{I/-}$ force; −/−, M-CK$^{-/-}$ force.

In contrast, two other types of alterations were evident in muscles with 3- and 6-fold reduced MM-CK levels. First, in both mutants, the exchange of high-energy phosphates between ATP and PCr was still below the level of ^{31}P NMR detection. Thus, in muscles with less than one-third of wild-type MM-CK activity, the NMR visible flux through the CK reaction is at least 20-fold below normal. Remarkably, the flux approximated normal values again when the enzyme levels increased from one-third to one-half of the normal MM-CK activity. A possible explanation for this phenomenon might be that ^{31}P NMR is only detecting the phosphoryl flux through MM-CK dimers in the sarcoplasm and not through the MM-CK fraction associated with the sarcoplasmic reticulum (15) and myofibrillar M and I bands (16, 17) or mitochondrial-bound Mi-CK (18). It is conceivable that saturation of these subcellular binding sites will not be achieved in mice with only 34% or less MM-CK levels. Consequently, levels of soluble MM-CK may be too low to catalyze a NMR visible exchange between PCr and ATP. On the other hand, in M-CK$^{+/-}$ muscles, there may be sufficient free dimers to nearly restore the CK flux. Interestingly, the CK flux has been reported to increase 2-fold in muscles of transgenic mice expressing soluble brain CK dimers (19). This supports the idea that NMR visibility of fluxes is dependent on the level of unbound CK isoenzymes.

The notion that homozygous or heterozygous combinations of M-CK alleles with <50% residual activity resulted in significantly reduced muscle burst performance (Fig. 3) is also of major physiological relevance. Our data show that graded reduction of muscle MM-CK activity is accompanied by an increasing inability to maintain maximal muscle force during a series of initial contractions. Noteworthy is also that the sudden drop of muscle force in M-CK$^{I/-}$ and M-CK$^{I/I}$ mutants occurred within the first eight muscle contractions, as in null mutants. Since local ATP/ADP ratios are known to influence muscle force (1, 3), the most straightforward explanation for our findings might be that the rate of ATP replenishment during high-intensity exercise is affected differently in the various genotypes because of different amounts of MM-CK associated with the myofibrillar M line (4, 20). To test this idea, further studies into the subcellular distribution of MM-CK in our set of M-CK mutants with graded reduction of enzyme activity will be required.

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