Altered $\text{Ca}^{2+}$ Responses in Muscles with Combined Mitochondrial and Cytosolic Creatine Kinase Deficiencies


The Netherlands

Summary

We have blocked creatine kinase (CK)-mediated phosphocreatine (PCr) $\rightarrow$ ATP transphosphorylation in skeletal muscle by combining targeted mutations in the genes encoding mitochondrial and cytosolic CK in mice. Contrary to expectation, the PCr level was only marginally affected, but the compound was rendered metabolically inert. Mutant muscles in vivo showed significantly impaired tetanic force output, increased relaxation times, altered mitochondrial volume and location, and conspicuous tubular aggregates of sarcoplasmic reticulum membranes, as seen in myopathies with electrolyte disturbances. In depolarized myotubes cultured in vitro, CK absence influenced both the release and sequestration of $\text{Ca}^{2+}$. Our data point to a direct link between the CK-PCr system and $\text{Ca}^{2+}$-flux regulation during the excitation and relaxation phases of muscle contraction.

Introduction

Creatine kinases (CKs; EC 2.7.3.2) form a small family of isoenzymes that help in keeping cellular ATP/ADP ratios delicately balanced and the ATP pool highly charged. Different CK isofoms exist in mitochondria (dimer octameric sarcomeric and ubiquitous mitCKs) and cytosol (homo- and heterodimeric MM-, BM- and BB-CKs), and occur in a strictly cell type-dependent manner in tissues with large fluctuations in energy metabolism, such as muscle and nerve (Wallimann et al., 1992). Although many concepts have been formulated to explain the involvement of the CK reaction [phosphocreatinase $\text{PCr}$ + MgADP$^-$ + H$^+$ $\rightarrow$ creatine [Cr] + MgATP$^2-$] in high energy phosphoryl homeostasis (Bessmann and Carpenter, 1985; Wallimann et al., 1992), little is known about its actual role in maintaining the integrity of metabolic energy compartmentalization (Miller and Horowitz, 1986; Saks et al., 1994) and the communication between intracellular sites of ATP consumption and production. It is generally accepted that the CK system helps in setting local concentrations and ratios of ATP and its hydrolysis products ADP, AMP, P$_i$, and H$^+$. In turn, these parameters govern the dynamic kinetics of many ATPases in different microenvironments (Rossi et al., 1990; Korge and Campbell, 1994) and may determine the rate of glycolysis and oxidative phosphorylation (OXPHOS; Erecinska and Wilson, 1982; Balaban, 1990). However, flux distribution of high energy phosphoryl metabolites in different cellular compartments is intriguingly complex and may involve the intertwining of interconversion reactions catalyzed by CKs, adenylate kinases (AKs), nucleoside mono- and diphosphate kinases, hexokinase, or glycerol kinase (Brdiczka and Wallimann, 1994). Individual reaction steps in this complex scheme might well be facilitated by the subcellular clustering of these kinases in multienzyme complexes (Bessmann and Carpenter, 1985).

To assess the importance of CK-catalyzed reactions in this cellular energy network of skeletal muscle, we have generated mice lacking both the mitochondrial CK (ScCKmit) and cytosolic CK (M-CK) by interbreeding ScCKmit$^{-/-}$ (Steeghs et al., 1996) and M-CK$^{-/-}$ (van Deursen et al., 1993) mice, and studied the phenotypic consequences in whole muscles in vivo and in cultured myotubes in vitro. We show that the animals are apparently normal but exhibit changes in mitochondrial morphology and composition, contain a hyperproliferative sarcoplasmic reticulum (SR), and show severely impaired force generation. In addition, we found that cultured myotubes exhibit an altered $\text{Ca}^{2+}$ response upon depolarization. Our results suggest that the CK system has a direct role in the regulation of both $\text{Ca}^{2+}$ accumulation and release in skeletal muscle fibers.

Results

Generation of Mice with Combined M-CK and ScCKmit Deficiency

To test whether skeletal muscle development and functioning depend on the continuous access to high energy phosphoryls provided by PCr, we interbred ScCKmit$^{-/-}$...
Page 94

and M-CK~'/~ mice to create a loss of function at both the mitochondrial and cytosolic end points of the PCr-CK system. CK~'/~ double mutants were identified in offspring from intercrosses between heterozygous ScCKmit~'/~; M-CK~'/~ animals at approximately the numbers predicted for normal Mendelian segregation with unlinked genes (10 out of 215). Contrary to expectation, they were not overtly different from wild-type or heterozygous littermates, bred normally, and had a normal life expectancy. Zymogram assays confirmed the complete absence of muscle-type CK activity and revealed that there was no ectopic expression of any of the other CK family members, i.e., the brain-type (B-CK) or the ubiquitous mitochondrial CK (UbCKmit) (Figure 1). Residual total CK activity in heart and skeletal muscle extracts was lowered to 0.3% and 2% of wild-type levels, respectively, and can be entirely attributed to the BB-CK and UbBCKmit content in capillary or vasculature endothelium and muscle satellite cells. These results provide evidence that basal muscle physiology is not dramatically affected by the lack of CK activity.

**Table 1. Concentrations of Energy Metabolites in CK-Mutant Muscle**

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>ScCKmit~'/~</th>
<th>M-CK~'/~</th>
<th>CK~'/~</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi (NMR)</td>
<td>5.6 ± 0.9</td>
<td>4.8 ± 0.8</td>
<td>4.5 ± 0.8</td>
<td>8.7 ± 0.5*</td>
</tr>
<tr>
<td>PCr (NMR)</td>
<td>46.5 ± 1.3</td>
<td>46.9 ± 0.7</td>
<td>47.3 ± 1.2</td>
<td>35.6 ± 1.6*</td>
</tr>
<tr>
<td>PCr (chem)</td>
<td>62.1 ± 2.2</td>
<td>64.9 ± 2.6</td>
<td>66.7 ± 3.0**</td>
<td>51.6 ± 5.2**</td>
</tr>
<tr>
<td>Cr (chem)</td>
<td>31.9 ± 2.2</td>
<td>29.1 ± 2.8</td>
<td>25.3 ± 3.0**</td>
<td>42.4 ± 5.2**</td>
</tr>
<tr>
<td>PCR/Cr (chem)</td>
<td>1.96 ± 0.20</td>
<td>2.26 ± 0.32</td>
<td>2.76 ± 0.45**</td>
<td>1.24 ± 0.27**</td>
</tr>
<tr>
<td>α-ATP (NMR)</td>
<td>16.7 ± 0.9</td>
<td>17.5 ± 0.7</td>
<td>16.7 ± 0.6</td>
<td>17.8 ± 1.0</td>
</tr>
<tr>
<td>β-ATP (NMR)</td>
<td>15.4 ± 0.5</td>
<td>15.5 ± 0.8</td>
<td>14.0 ± 0.4</td>
<td>17.0 ± 0.7*</td>
</tr>
<tr>
<td>γ-ATP (NMR)</td>
<td>14.1 ± 0.8</td>
<td>14.5 ± 1.1</td>
<td>13.7 ± 0.6</td>
<td>16.1 ± 0.6*</td>
</tr>
<tr>
<td>Mean ATP (NMR)</td>
<td>15.4 ± 0.3</td>
<td>15.8 ± 0.4</td>
<td>14.8 ± 0.3</td>
<td>17.0 ± 0.6*</td>
</tr>
<tr>
<td>ATP (chem)</td>
<td>24.5 ± 4.8</td>
<td>22.8 ± 2.0</td>
<td>19.1 ± 2.1</td>
<td>18.1 ± 2.6*</td>
</tr>
<tr>
<td>PCR/ATP (NMR)</td>
<td>3.03 ± 0.15</td>
<td>2.96 ± 0.08</td>
<td>3.20 ± 0.14</td>
<td>2.11 ± 0.18*</td>
</tr>
<tr>
<td>PCR/ATP (chem)</td>
<td>2.93 ± 0.57</td>
<td>2.85 ± 0.24</td>
<td>3.65 ± 0.49</td>
<td>2.88 ± 0.24</td>
</tr>
<tr>
<td>P_7/PCr (NMR)</td>
<td>0.12 ± 0.02</td>
<td>0.10 ± 0.02</td>
<td>0.09 ± 0.02</td>
<td>0.25 ± 0.02*</td>
</tr>
<tr>
<td>PK (NMR)</td>
<td>2.7 ± 0.7 (4)</td>
<td>2.0 ± 0.2 (2)</td>
<td>3.8 ± 0.9 (5)</td>
<td>4.8 ± 1.0 (6)</td>
</tr>
</tbody>
</table>

Concentrations were either determined by chemical analysis of snap-frozen medial gastrocnemius muscle (given as mean number of μmol/g dry weight ± SD) or calculated from the relative peak areas of phosphate metabolites in fully relaxed NMR spectra (given as percentage of total integral, with [ATP] as average of the three peaks) of whole hind-limb skeletal muscle of wild-type and CK-mutant mice. Cr and Pcr concentrations were normalized for the mean total Cr + Pcr content of all muscles. Ratios calculated from the chemical determination and NMR measurements are given individually. In between brackets are the numbers of experiments when the phosphomonoester (PME) signal was clearly above the noise level. The total number of determinations was n = 8 for wild-type and CK~'/~; n = 6 for M-CK~'/~ animals, and n = 4 or n = 6 for chemical or NMR determination of ScCKmit~'/~ animals, respectively. Double asterisks and single asterisks denote significant differences (p < 0.01 and p < 0.02, respectively) between the wild-type group and each of the CK mutants.

**Figure 1. Zymogram Gel Analysis of CK-Mutant Muscles**

Activity staining of the different native CK isoenzymes in extracts from skeletal (lanes 1-4) and heart (lanes 5-8) muscle from wild-type (+/+) (lanes 1 and 5), M-CK~'/~ (lanes 2 and 6), ScCKmit~'/~ (lanes 3 and 7), and CK~'/~ double-mutant (lanes 4 and 8) mouse. Positions of CK isoenzymes and residual AK activity (noninhibited by AP5A) are indicated. Note the complete absence of CK activity in (−/−) skeletal muscles.
Creatine Kinases Modulate Ca²⁺ Homeostasis

A

PCr

ATP

β

γ

α

Pi

5 Hz

5 Hz

1 Hz

1 Hz

0

5

10

15

-15 ppm

-10

-5

0

C

D

PCr

ATP

Pi

12

10

8

6

4

2

0

-2

-4

-6

-8

-10

-15 ppm

5 Hz

5 Hz

1 Hz

1 Hz

Figure 2. ³¹P-NMR Recordings of Changes in Phosphate Metabolites during Muscle Stress

Each spectrum ([A], wild type; [B], CK⁻⁻⁻⁻ mutan; 18 free induction decays of 70°C pulses, 5 s repetition time) was obtained from lower limb muscle at rest, followed by 8 min of stimulation at 1 Hz, 8 min of recovery, 8 min of stimulation at 5 Hz, and finally, 6 min of rest. (C) (wild type) and (D) (CK⁻⁻⁻⁻) (spectra acquired with a scan repetition time of 12 s) show the course of changes in levels of phosphorylated metabolites before (bottom spectra) and during complete anoxia (cardiac arrest) of the animals. Pi, PCr, and the α-, β-, and γ-ATP peaks are indicated; the PCr peak was set at 0 ppm.

for COX and CS). Evidently, loss of the M-CK isoform is dominant, and additional loss of ScCKmit has no synergistic effects on the mitochondrial machinery.

Next, we applied in vivo ³¹P-NMR to monitor energy conversion processes in intact muscle. Essentially, no differences were observed for fully relaxed ³¹P-NMR spectra from hind-limb muscles of anesthetized wild-type and single-mutant mice. Resting-state spectra of ScCKmit⁻⁻⁻⁻ muscles confirm the unanticipated presence of PCr, as determined by chemical analysis (Figure 2B). A clear peak appears exactly at the chemical shift position expected for PCr (at 2.44 ± 0.02 ppm from the γ-ATP position) in spectra of CK⁻⁻⁻⁻ mice. The relative positions of the three ATP resonances in double-mutant skeletal muscle spectra are exactly similar to those in wild-type spectra. Relative peak areas of energy metabolites in ScCKmit⁻⁻⁻⁻ and M-CK⁻⁻⁻⁻ single mutants are also similar to those in wild type (Table 1), whereas CK⁻⁻⁻⁻ mutant muscles show a substantially lower PCr signal (35.6 versus 46.5-47.3) and somewhat higher ATP signals with respect to the total phosphate signal area. Consequently, this results in a 30% lower PCr/ATP ratio. The peak area measurements also show that relative levels of inorganic phosphate (Pi) and phosphomonooesters (PME) have increased significantly in CK⁻⁻⁻⁻ muscles, suggesting a rewiring of metabolism via the glycolytic route. Finally, it is important to note that pH calculations yielded similar values for wild-type (7.29 ± 0.07), ScCKmit⁻⁻⁻⁻ (7.17 ± 0.05), M-CK⁻⁻⁻⁻ (7.24 ± 0.02) and CK⁻⁻⁻⁻ (7.21 ± 0.05) mutant muscles.

PCr Is Not Hydrolyzable in CK⁻⁻⁻⁻ Muscles

The presence of PCr in CK⁻⁻⁻⁻ muscles at rest raised the question as to whether this reservoir could still serve as a temporal energy buffer at the onset of exercise. Changes in levels of ³¹P energy metabolites were therefore monitored during and after isometric contraction of lower hind-limb muscle. PCr, Pi, and ATP profiles of wild-type muscle recorded during periods of stimulation at 1 or 5 Hz and during subsequent recuperation periods were as expected (Figure 2A). In CK⁻⁻⁻⁻ muscles, levels of PCr and ATP remained apparently constant during and after the exercise period (Figure 2B). Interestingly, also after prolonged exercise periods (20 min; 5 Hz), no significant changes in peak areas of the 3 phosphates of ATP were observed, while intracellular pH responses were comparable to those in wild-type muscle within the time resolution of the experiment. Finally, phosphate metabolites were also monitored under conditions of prolonged severe anoxia. In skeletal muscle of wild-type mice (and ScCKmit⁻⁻⁻⁻ or M-CK⁻⁻⁻⁻ mice; data not shown), PCr hydrolysis with a simultaneous fast and steep increase in Pi levels preceded ATP utilization (Figure 2C). In contrast, the level of PCr in CK⁻⁻⁻⁻ muscle remained stable, while the complete hydrolysis of ATP occurred with an increase of Pi (Figure 2D), indicating
that high phosphoryl PCr → ATP exchange is impossible.

CK Deficiency Reduces Muscle Force Development

Energy consumption in normal muscle at work is a summation of highly local ATP-hydrolysis events in individual fibers, predominantly supporting SR Ca2+ pump (SERCA) and Na+/K+ pump activity and cross-bridge cycling for acto-myosin sliding. Since we wished to see whether these activities unite differently in a CK-deficient background, we recorded the contractile behavior of medial gastrocnemius muscles of mutant and control mice upon delivery of a fast and repetitive series of action potentials (producing a tetanus as the Ca2+ concentrations in the cytosol level off) [Table 2 and Figures 3A-3C]. Already in response to the second stimulus, and following all subsequent pulses, an impaired force production was seen for CK−/− compared with the wild-type muscle. The force started to decay significantly after the sixth stimulus (60 ms), whereas in wild-type (and ScCK−/−) muscles peak force was not reached before approximately 150 ms of stimulation. Lowering of tetanic force also resulted in lower tetanic/twitch force ratios for these mutants (Table 2). In an intermittently repeated isometric contraction series (Figure 3B), there was a clear difference in initial force production between the wild-type and ScCK−/− group on the one hand and the M-CK−/− and CK−/− group on the other hand. Whereas the force generated by the first repeated contractions initially increased in the wild-type and ScCK−/− muscles, a large drop in force production (±30%) already occurred in the second twitch of the series in M-CK−/− and CK−/− muscles. Thereafter, force in CK−/− muscles declined further, while M-CK−/− muscles were able to recover slightly. Half-relaxation times of the wild-type, ScCK−/−, and M-CK−/− muscles remained fairly constant during the first 10 twitches of the series of repeated contractions but increased by 3-5 ms thereafter. In contrast, half-relaxation time increased immediately after the first contractions in the CK−/− muscles, before leveling off later (Figure 3C). Although our measurements integrate all molecular effects on local ATP supply in the entire muscle, comprising different fiber types, our results are most adequately explained by slowed kinetics for Ca2+ release instead of uptake. Effects on Ca2+-ATPase-mediated sequestration of Ca2+ are not apparent in single mutants but—together with effects on the rephosphorylation of actin-myosin-bound ADP (Cooke and Pate, 1985; Sata et al., 1996)—may dominate the slower relaxation behavior of CK−/− double mutants. In a preliminary approach, we also studied whether there were changes in the myosin heavy chain (MHC) profile of CK-mutant myofibrils, because MHCs are the other principal determinants of shortening velocity and energetic economy of working muscle (Sweeney et al., 1988). As shifts in MHC isotype composition were relatively subtle and occurred in a complex mutation- and fiber type-dependent manner (Steeghs, 1995), they were further neglected in this study.

Cytoarchitectural Abnormalities of Structures Involved in ATP and Ca2+ Handling

Having demonstrated the physiological consequences of CK absence, we next examined whether conformational changes in mitochondria and the membranous structures for Ca2+ handling could provide a correlate for altered flux kinetics or compartmentalization of ATP or Ca2+. Ultrastructural and morphometric analysis of diaphragm, intercostal, soleus, gastrocnemius, and heart muscles revealed no changes in the arrangement of outer and inner mitochondrial membranes and cristae, not even in the number of mitochondrial contact sites in the two latter muscles in CK−/− mutants (L. Verdoort et al., submitted). In contrast, the enlarged intermyofibrillar mitochondrial volume that was apparent in fast-twitch fibers of M-CK-deficient gastrocnemius-psosas-soleus (GPS) muscles (van Deursen et al., 1993) was also observed in large fibers of GPS, diaphragm, and intercostal CK−/− muscles. Mitochondria in CK−/− muscles were often packed in rows, and their sizes were highly variable, ranging from extremely large (more than 5 μm in length) to very small. Moreover, mitochondria-rich fibers of all CK−/− muscles, including heart, diaphragm, and large glycolytic skeletal muscles, were distinctly different from other mutants and wild type, in that they contained large(r) numbers of lipid droplets (Figures 4C and 4D). In semithin 1 μm sections, these lipid droplets can often be seen as strings of beads. Electron microscopic examination revealed the lipid droplets to be located immediately adjacent to, and occasionally inside, the intermyofibrillar mitochondria. Association to the subsarcolemmal mitochondrial population was only rarely observed. Furthermore, mitochondria containing glycogen, lipofuscin granules, and other lysosomal structures were present in increased numbers. These deposits are common to diseased muscle and occur to a much lesser extent in normal muscle during aging (Dubowitz, 1985).

### Table 2. Force Characteristics of Medial Gastrocnemius Muscles of Wild-Type and CK-Mutant Animals

<table>
<thead>
<tr>
<th></th>
<th>Wild Type*</th>
<th>ScCK−/−</th>
<th>M-CK−/−</th>
<th>CK−/−</th>
<th>CK−/−</th>
<th>CK−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twitch force (N)</td>
<td>0.19 ± 0.05</td>
<td>0.17 ± 0.03</td>
<td>0.16 ± 0.03</td>
<td>0.17 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetanic force (N)</td>
<td>0.82 ± 0.22</td>
<td>0.78 ± 0.19</td>
<td>0.55 ± 0.04**</td>
<td>0.56 ± 0.25*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetanic/twitch ratio</td>
<td>4.28 ± 0.36</td>
<td>4.57 ± 0.40</td>
<td>3.41 ± 0.48**</td>
<td>3.39 ± 0.39**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to peak force (ms)</td>
<td>142 ± 4</td>
<td>142 ± 4</td>
<td>72 ± 12**</td>
<td>56 ± 7**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Half-relaxation time (ms)</td>
<td>5.0 ± 0.5</td>
<td>5.0 ± 0.5</td>
<td>7.2 ± 0.5**</td>
<td>7.5 ± 1.3**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Double asterisks and single asterisk, see Table 1. *n = 14, **n = 5, *n = 4, **n = 15.
Creatine Kinases Modulate Ca\(^{2+}\) Homeostasis

**Figure 3. Contractile Characteristics of Wild-Type and CK-Mutant Muscles**

(A) Tetanic and twitch force production in wild-type (full trace) and CK\(^{−/−}\) muscles (dotted trace). Note the similar twitch force in both muscles.

(B) Changes in isometric force production during a series of 20 repeated contractions (intermittent isometric protocol). Successive contractions (intermittent isometric protocol) were repeated contractions in wild-type (open squares, 5), and CK\(^{−/−}\) (closed circles, n = 5), and CK\(^{+/+}\) (open circles, n = 6) muscles. Forces (mean ± SEM) are expressed as a percentage of the force in the first contraction of each series.

(C) Changes in half-relaxation times in a series of 20 repeated isometric contractions. Denotations and numbers of experiments are similar as in (B), except for ScCKmut\(^{+/−}\) where n = 5. Half-relaxation times are mean ± SEM.

Omnipresence of conspicuous darkly staining elongated inclusions within the large fibers of gastrocnemius and intercostal muscles in semithin sections is another distinct feature of CK\(^{−/−}\) animals (Figure 4E). Within the fibers, the inclusions are seen at both subsarcolemmal and intermyofibrillar localizations. Ultrastructural examination of various fibers revealed that the inclusions consist of closely packed longitudinally oriented clusters of membranes known as tubular aggregates (TAs) (Figures 4F and 4G). Besides the well-ordered aggregates, unorganized more dilated membrane structures with varicosities containing electron-gray material were also observed (Figure 4H). TAs are a distinct pathological structure in skeletal muscle consisting of aggregated terminal cisternae or longitudinal components of the SR. Figure 4I shows that cisternae of the SR, which are located in the direct surroundings of connexions with tubular inclusions, are indeed heavily dilated. The myofibrillar compartment adjacent to the tubular aggregates showed no structural abnormalities, and triads in those myofibrils also appeared normal. It has been suggested that tubular morphology of longitudinal SR in mature muscle arises as a result of reshuffling of SERCA isoforms (Martonosi, 1996). To test whether a chronic derangement in SERCA content could underly TA formation, we measured its total level in extracts of hind-leg muscles of CK mutants. Semiquantitative immunoblot analysis and in vitro biochemical assays revealed that Ca\(^{2+}\)-ATPase activity (76.5 ± 9.5 in wild type, 75.5 ± 9.8 in M-CK\(^{−/−}\)-, and 76.6 ± 10.0 mU/mg protein in CK\(^{−/−}\)-mutants) and content (between 94.3 ± 11.0 and 95.2 ± 11.8 pmol/mg protein) were similar, regardless of whether the muscles expressed CKs or not. Also, the levels of calsequestrin, another SR marker, remained constant in all muscle types. For reasons unknown, the content of protein disulphate isomerase (PDI), an SR-lumen marker, was slightly increased in CK\(^{−/−}\)-muscles only (Figure 5H). Thus, if SERCAs play a role in the conversion of SR cisternae into TAs, it is most likely the redistribution rather than an overall higher membrane content of these pumps that is involved.

**Ca\(^{2+}\) Transients Are Altered in CK-Mutant Myotubes In Vitro**

Since all (patho)histological and physiological changes in vivo are most easily explained by effects on the Ca\(^{2+}\)-handling machinery, we were prompted next to compare Ca\(^{2+}\) homeostasis in skeletal muscle cells in culture. To this end, satellite cells were isolated from wild-type and mutant animals, and the derived primary myoblasts fused to myotubes in culture. The free cytosolic [Ca\(^{2+}\)] and shape of the transients were measured by digital imaging of single Fura 2-loaded myotubes (Benders et al., 1994). The graphs in Figure 5 show averaged traces of the Ca\(^{2+}\) levels at rest and the Ca\(^{2+}\) transients resulting from acetylcholine- (ACh; 20 µM) or KCl- (125 mM) stimulated depolarizations. The basal [Ca\(^{2+}\)] level under standard physiological conditions was similar in wild-type and mutant skeletal muscle cells. In contrast, at depolarization by ACh or KCl, the amplitude of the Ca\(^{2+}\) responses ([Ca\(^{2+}\)]\textsubscript{plateau}) was decreased by about 30% in mutant myotubes, whereas the plateau level ([Ca\(^{2+}\)]\textsubscript{plateau}) after stimulation was increased by 35% and 58% in M-CK\(^{−/−}\) and CK\(^{−/−}\) cells, respectively. Moreover, both the half-increase (t) and the half-decay time...
(\(t_a\)) of the Ca\(^{2+}\) transients were about 1.3- to 1.5-fold higher in the mutant muscle cells. To determine whether the Ca\(^{2+}\) release pathways were the same in all 3 cell types, we examined the effects of nifedipine (5 \(\mu\)M) and ryanodine (10 \(\mu\)M), i.e., specific inhibitors of the voltage-dependent L-type Ca\(^{2+}\) channel (dihydropyridine receptor) and SR Ca\(^{2+}\) release channel (ryanodine receptor), respectively. Both agents completely abolished the depolarization-induced Ca\(^{2+}\) responses (Figures 5C-5F) in all cell types. We interpret this to indicate that there are no other Ca\(^{2+}\) channels effective in our mutants than in the wild type. Likewise, as the SERCA activity and content (13.0 ± 2.6, 12.9 ± 4.8, and 13.6 ± 3.1 mU/mg protein with content ranging between 16.1 ± 3.2 and 17.0 ± 4.0 pmol/mg protein for wild type, M-CK\(^{−/−}\), and CK\(^{−/−}\), respectively), as well as the calasequestrin and PDI levels (Figure 5G), were essentially identical between cell types, we can almost rule out gross alterations of SR composition. Taken together, our results suggest that M-CK deficiency is the direct governing principle in the observed changes in the magnitude and velocity of the Ca\(^{2+}\) fluxes, as various parameters were equal in M-CK\(^{−/−}\) and CK\(^{−/−}\) cells.

Discussion

The primary observations presented in this paper provide evidence that the CK-PCr system, which is directly coupled to energy transduction, is also intimately connected to the processes for Ca\(^{2+}\) release and storage in skeletal muscle. Unexpectedly, although ATP and Ca\(^{2+}\) are crucial regulators of many physiological processes, we observed no outwardly overt effects on the physiological functioning of voluntary or nonvoluntary muscles in CK\(^{−/−}\) mice. This raises the issue of the fate of metabolism and ion homeostasis in the absence of CK.

Redirection of pathways for synthesis and usage of ATP within a muscle fiber may primarily lead to local displacement of AMP/ADP/ATP ratios, change local Mg\(^{2+}\), H\(^{+}\), and inorganic phosphate (P) concentrations, and affect—more indirectly—inosine monophosphate
and lactic acid levels. In turn, these alterations may translate into adaptational shifts in the oxidative or glycolytic metabolic profiles, depending on fiber type (Ventura-Clapier et al., 1995; O’Gorman et al., 1996). Indeed, in our mice with single CK deficiencies, differential compensatory consequences on carbohydrate metabolism were seen in fast-twitch muscle having a relatively high proportion of cytosolic MM-CK and in slow-twitch oxidative muscle being particularly rich in ScCKmit. For single ScCKmit−/− mice, it was previously postulated (Steeghs et al., 1996) that high energy phosphoryl conversions mediated by MM-CK, a process that could involve a relocation of MM-CK to sites around the mitochondrial periphery. ATP from OXPHOS can (and most likely also does) reach the cytosolic compartment independently of the ScCKmit reaction in this case. Conversely, for situations where there is only a single deficiency of the cytosolic M-CK, we had postulated that PCR can be redirected through the mitochondria and contribute to ATP production in situations of acute energy demand (van Deursen et al., 1993, 1994). To demonstrate the efficacy of our double-knockout strategy, it was therefore first necessary to determine if PCR metabolism was rendered unfunctional. Unexpectedly, chemical analysis and NMR assays revealed PCR to be present in muscles of all kind. To explain this observation, we have considered several possibilities: (i) a complete picture, further study of PCR levels in myofibrils is warranted to see if and at what age a steady-state situation in accumulation is reached.

By use of in vivo NMR analysis, however, we could confirm that PCR no longer can serve to regenerate hydroyzed ATP in CK−/− double mutants, not even under the most strenuous situations (i.e., complete anoxia). The absence of CK-catalyzed phosphotransfer in muscle of CK−/− mutants has been confirmed by monitoring

Figure 5. Ca²⁺ Transients in Skeletal Muscle Cells
Depolarization was induced in differentiated myotubes from different control or mutant mice by adding 20 μM ACh (A, C, and E) or changing the KCl concentration to 125 mM (B, D, and F). Traces show the averaged [Ca²⁺]i after superimposing all appropriate experiments for wild-type (solid line), M-CK−/− (dashed line), and CK−/− (dotted line) myotubes; numbers examined are given between parentheses. The basal [Ca²⁺]i in myotubes derived from wild-type, M-CK−/−, and CK−/− mice is 122 ± 17 nM (156), 122 ± 10 nM (65), and 126 ± 22 nM (86), respectively. Mean values ± SD for the amplitude ([Ca²⁺]max), or plateau of the Ca²⁺ responses ([Ca²⁺]plateau) are expressed in nM, and the half-increase (τa) or half-decay time (τd) in seconds. Parameters of mutant myotubes differ from those of wild-type myotubes with p < 0.01 (asterisks) or from each other with p < 0.01 (pound signs). The effects on depolarization of presence of the dihydropyridine receptor channel-blocker nifedipine (C and D) or the ryanodine receptor channel-blocker ryanodine (E and F) are shown in the middle panels. The bottom panels show immunostained signals from SR marker enzymes, SERCA1, calsequestrin, and PDI on strips of Western blots of cultured muscle cells (G) and adult skeletal muscle (H). SERCA1 and calsequestrin signals are present in all lanes (40 μg protein/lane, except for M-CK−/− cells, 20 μg/lane) but occur at different levels in cell and muscle extracts (note the overstained SERCA signals in all muscle types). Note that the PDI signal is of equal intensity in all lanes but absent in wild-type and M-CK−/− muscle extracts.
the rates of \(^{18}\text{O}\)-phosphoryl appearance in endogenous metabolites, including PCR, of muscle incubated in \(^{18}\text{O}\)-water-enriched medium (N. D. Goldberg, personal communication). Taken together, we consider this strong evidence for all escape routes in ATP \(\rightarrow\) PCR conversions being essentially blocked, if we combine nulligygous mutations in both end points of the CK–PCR shuttle. Equally important, these data confirm that our previous postulates about redirection of phosphoryl fluxes in single CK mutants were essentially correct. Combined with the observation that PCR can indeed be converted into ATP in the livers of UbCKmit transgenic mice (Koretsky, 1995), our results strongly suggest that the direction of the mitochondrial and cytosolic energy flux in vivo (i.e., toward production or consumption of PCR) is entirely dependent on the physiological state of the cell.

We conclude that the remarkable preservation of basal physiological functions in CK\(^{--}\) muscles must be based on otherwise cryptic principles of metabolic control and energy homeostasis. Zelenskii et al. (1995) have provided compelling evidence that AK-mediated ATP \(\rightarrow\) ADP exchange is likely involved, as it can partially take over the metabolic energy transfer function. Almost certainly, the fiber type-dependent changes in mitochondrial location and the glycolytic energy flow are other important determinants in the long-term adaptive process in our mutants. An increase in intermyofibrillar—not but subsarcolemmal—mitochondrial volume in fast-twitch fibers was evident in both CK\(^{--}\) and CK\(^{+}\) mutants, and was described earlier (van Deursen et al., 1993). This phenomenon may serve to preserve adequate ATP delivery and protect the system against kinetically unfavorable substrate-product feedback, which would occur if transcytotic gradients of ATP, ADP, and P\(_i\) were simply increased (Gunter et al., 1994). When the overall CK\(^{--}\) muscle ultrastructure is compared with that of CK\(^{+}\) muscles, however, conspicuous additional pathological manifestations become evident. The presence of lipofuscin granules points to deregulation of metabolism, and the accumulation of lipid in and around the mitochondria in CK\(^{--}\) mutants may indicate an impaired capacity to utilize fatty acids as substrate for oxidative ATP production (DiDonato et al., 1978). We may expect that unraveling the functional nature of these phenomena will provide more insight into the role of the mitochondrial end of the CK system and its connection to distributed metabolic control. There is now compelling evidence that mitochondrial Ca\(^{2+}\) is the most likely candidate for an additional metabolic mediator (Gunter et al., 1994; Hajnóczky et al., 1995), but future studies are necessary to confirm the suggestion that ATP–PCr metabolism and Ca\(^{2+}\) homeostasis indeed converge in mitochondria.

In sarcoplasm, [Ca\(^{2+}\)], exerts a dominant regulatory control on the excitation–contraction coupling (Ebashi, 1976) and acts as a second messenger to modulate cellular function. The proposal of local Ca\(^{2+}\) alterations being the principal reporter and regulator for metabolic, physiological, and cytoarchitectural changes in CK-deficient muscles is therefore consistent with the observation that M-CK\(^{--}\) and CK\(^{+}\) mutants, besides ultrastructural features, share striking similarities in tetanic force profiles and Ca\(^{2+}\)-release/uptake characteristics in vivo and in vitro. Based on the idea that the altered CK activity influences [Ca\(^{2+}\)], the quick and significant drop in force generation seen in intermittently stimulated M-CK\(^{--}\) and CK\(^{+}\) muscles can be most easily explained by a drop in the magnitude of Ca\(^{2+}\) release and a lesser recruitment of fibers (Edwards et al., 1975; de Haan et al., 1989). Comparison of the Ca\(^{2+}\) transients in cultured wild-type and mutant myotubes suggests a more complicated model, whereby the response amplitude, speed of release (\(r_\text{s}\)), and sequestration (\(r_\text{q}\)) of Ca\(^{2+}\) have been changed. To date, little is known regarding the reliability of extrapolating data from the in vitro to the in vivo situation, especially as the cytoarchitectural arrangement of the plasma membrane, myofilaments, and SR vesicles in muscle cells may differ from the situation in vivo (Flucher and Franzini-Armstrong, 1996). We observed that the depolarization-induced Ca\(^{2+}\) transients are prevented by inhibition of the Ca\(^{2+}\) channels in both cultured wild-type and mutant muscle cells (Figures 5E and 5F), suggesting that a "skeletal muscle type" of excitation–contraction coupling mechanism is operational. Likewise, we know that a similar mechanism for uptake of Ca\(^{2+}\) in sequestering SR vesicles exists in developing and adult fibers, underscoring the validity of comparing in vitro and in vivo findings. Still, for an adequate interpretation of our results it is important to stress that our cultured myotubes contain a significant level of BB-CK activity, the early embryonic CK isofrom in muscle. On average, 28% \(\pm\) 10% (n = 10) of total CK activity in cultured wild-type cells resulted from BB-CK activity, while the absolute level of BB-CK in mutant cells was similar to that in wild type. We thus have to conclude that BB-CK cannot fully substitute MM-CK in counteracting [Ca\(^{2+}\)] changes at depolarization, although this is not relevant for in vivo physiology per se, as B-CK expression is switched off completely during development (Wallimann et al., 1992) (Figure 1).

What could be the mechanisms by which CK absence affects both the amplitude and kinetics of release and uptake in Ca\(^{2+}\) transients? Impaired PCR \(\rightarrow\) ATP exchange could influence the speed and magnitude of Ca\(^{2+}\) release by setting a local change in the cytosolic ([Ca\(^{2+}\)]) and SR luminal [Ca\(^{2+}\)], owing to impaired SERCA action. In turn, this may affect the open probability (P\(_o\)) of the ryanodine receptor and thus influence quantal Ca\(^{2+}\) release from the SR (Hidalgo and Donoso, 1995; Klein et al., 1996). ATP availability can also influence the P\(_o\) more directly. Local depletion of [ATP], and the accompanying increase in free [Mg\(^{2+}\)] near the channel, can lead to a reduction in Ca\(^{2+}\) release in skeletal muscle fibers (Owen et al., 1996) and influence the filling state of the internal Ca\(^{2+}\) stores (Hofer et al., 1996). Furthermore, local changes in [ATP] could induce changes in the protein phosphorylation status and thus the activities of dihydropyridine receptor and ryanodine receptor clusters (Valdivia et al., 1995). It is also conceivable that the function of the Na\(^+/K^+\) pump (Wallimann et al., 1992) and/or ATP-sensitive potassium channels (Olson et al., 1996) in the sarcolemma membrane might be severely disregulated by alterations in local [ATP] and affect depolarization characteristics in cells and muscles alike. Clarifying whether one or a combination of these factors is involved represents an important issue for future investigations.
Finally, there is more consistent information on the possible mechanisms for altered Ca\textsuperscript{2+} uptake. Our work extends evidence suggesting that the efficiency of uptake (i.e., SR storage) of Ca\textsuperscript{2+}, in which different SERCAs are the key players (Pozzan et al., 1994), is significantly improved by the presence of membrane-bound MM-CK acting as a local ATP regenerator (Rossi et al., 1990; Korge and Campbell, 1994; Minajeva et al., 1996). Although we do not know the physical basis for this association, we assume that MM-CK can provide preferential access to ATP to all SERCA isoforms in different muscle fiber types at different phases of maturation. As we saw no gross differences between mRNA levels for SERCA (data not shown) and protein content or activity (Figure 5) in our mutants, this suggests that the slowing of Ca\textsuperscript{2+} uptake in M-CK\textsuperscript{i--} or CK\textsuperscript{1/2} cells is indeed a direct effect of altered energetic P availability only, though additional effects of altered local [H\textsuperscript{+}] (Donoso et al., 1996) cannot be excluded. SERCAs work near thermodynamic equilibrium at high \DeltaE, and since the \DeltaE of ATP hydrolysis is significantly lower than that of Pcr (Wallmahl and Martonosi, 1992), Ca\textsuperscript{2+} pump activity could be readily perturbed if access to Pcr is denied. With regard to effects of architectural changes in SR vesicles, it is noteworthy that the appearance of tubular aggregates (TAs) was unique to CK\textsuperscript{i/2} double mutants, and therefore not crucial for the Ca\textsuperscript{2+}-related effects shown in Figures 3 and 5. TAs occur mainly in type II fibers and may be functionally equivalent to hypertrophy of the SR terminal cisternae, as they are critical for the calcium storage capacity (Salvati et al., 1985) and have been shown to be highly reactive with antibodies against Ca\textsuperscript{2+}-ATPases and calsequestrin. In this study, formation of TAs was only evident in whole muscle and not in cells in vitro, and CK mutations did not seem to influence the level of TA marker enzymes. Remarkably, TALike structures have not been reported in creatine-depleted muscles when rodents are fed with creatine analogs. In humans, the occurrence of TAs has been associated with (genetic) disorders with electrolyte disturbances (Rosenberg et al., 1985; Hoffman et al., 1995).

In mice, TAs have been observed in murine dystrophy heterozygotes (Craig and Allen, 1980) and in the MRL\textsuperscript{+/+} substrain (Kunccl et al., 1989). From study of the latter model, it is clear that their appearance is genetically predisposed and gender (i.e., hormone) and age related. TAs occur close to the T-tubule-SR triadic junction, an area that is also a site of action of compartmentalized glycolytic activity (Han et al., 1992) and a site where part of M-CK is localized (T. Wallmahl, personal communication). Most interestingly, there is evidence that interactions between Ca\textsuperscript{2+}-ATPase molecules are principal organizing forces that determine the tubular shape of longitudinal SR vesicles, and that P, and Ca\textsuperscript{2+} can modulate these interactions (Martonosi, 1996). Together, these observations suggest that only in CK\textsuperscript{i/2} myotubes in vivo is there sufficient imbalance in local concentrations of phosphoryl metabolites, P\textsubscript{n}, H\textsuperscript{+}, or Ca\textsuperscript{2+} to trigger conformational changes in SR vesicles, possibly involving a redistribution of SERCA and calsequestrin molecules.

In summary, our experimental approach has allowed us to address the CK-PCr system in an in vivo context. Our data confirm predictions based on in vitro studies and provide more general evidence for a specific and direct functional coupling between MM-CK, ScCkmit, and the Ca\textsuperscript{2+}-handling machinery at stages of advanced muscle development. Coupling to M-CK and ScCkmit may tune the regulation of Ca\textsuperscript{2+} release, as well as the rate of Ca\textsuperscript{2+} sequestration in cytosolic and mitochondrial compartments, and does so differently in different muscle fibers. In turn, the compartmentalized (Ca\textsuperscript{2+}) may act as a second messenger in adapting the cellular organization to physiological demand. Further analysis of the embedding of CK protein and the catalyzed phosphoryl transfer reaction in different microenvironments under different physiological challenges is necessary to understand the details on the coupling of PCR-ATP supply and these spatial and temporal events in Ca\textsuperscript{2+} homeostasis in muscle.

Experimental Procedures

Generation and Genotyping of CK-Deficient Mice

Procedures involved in the generation and genotyping of M-CK\textsuperscript{i--} and ScCkmit\textsuperscript{i--} nullizygous mice have been described in detail elsewhere (van Deursen et al., 1993; Steeghs et al., 1996). ScCkmit\textsuperscript{i--} and M-CK\textsuperscript{i--} mice were crossed to obtain animals heterozygous for both CK isoforms. Seyling matings of these mice resulted in the generation of ScCkmit and M-CK double-mutant CK\textsuperscript{i--} mice. All wild-type and CK-mutant mice had a mixed inbred (50%-50% C57BL/6 x 129/Sv) background. Breeding was carried out under standard housing conditions in the KUN Central Animal Facility.

CK Isoenzyme, Ca\textsuperscript{2+}-ATPase, and SR Marker Determination and Fiber Typing

Muscle extracts were prepared and diluted 1:5 in 30 mM NaPO\textsubscript{4} buffer (pH 7.4) containing 0.05% (v/v) Triton X-100, 0.3 mM dithiothreitol, and 0.3 mM phenyl methanesulphonyl fluoride (Steeghs, 1995). Total CK-specific activity was measured using a CAB-CK NAC-activated kit (Boehringer GmbH, Mannheim, Germany, No. 475742). For immunogrom analyses, extracts were diluted in Tris-barbital buffer (pH 8.8), and CK proteins (in 2.8-4.9 μg extract protein) were resolved on agarose gels using the Universal Gel 8 system from Ciba Corning (Alameda, CA). Enzyme activities were visualized using the creatine phosphokinase isoenzymes colorimetric detection kit (Sigma Diagnostic) according to the manufacturer’s instructions (Procedure no. 715-EP). Staining by AK activity was prevented by including 10 μM of the specific inhibitor P\textsuperscript{3},P\textsuperscript{4}-diadenosine-5'-penta- phosphosphate (Ap5A) in all staining mixtures.

For determining activity and concentration of SR Ca\textsuperscript{2+}-ATPase have been described previously (Benders et al., 1994). For visualization of SR constituents, extracts from muscles or cells (20-40 μg protein per lane) were fractionated on 7.5% (w/v) acrylamide–SDS gels and electrophoresed into nitrocellulose membranes. Separate strips of the blot were incubated with monoclonal antibodies specific for SERCA1 (1:5000 diluted 5D2; Benders et al., 1994), calsequestrin (1:1000; Sigma, St Louis, MO), and stained following routine methods. Electrophoretic analysis of MHC composition, and determination of the Ca\textsuperscript{2+} handling machinery at stages of advanced muscle development. Coupling to M-CK and ScCkmit may tune the regulation of Ca\textsuperscript{2+} release, as well as the rate of Ca\textsuperscript{2+} sequestration in cytosolic and mitochondrial compartments, and does so differently in different muscle fibers. In turn, the compartmentalized (Ca\textsuperscript{2+}) may act as a second messenger in adapting the cellular organization to physiological demand. Further analysis of the embedding of CK protein and the catalyzed phosphophoryl transfer reaction in different microenvironments under different physiological challenges is necessary to understand the details on the coupling of PCR-ATP supply and these spatial and temporal events in Ca\textsuperscript{2+} homeostasis in muscle.

Chemical Analysis of Metabolite Concentrations

Procedures for the measurements of creatine and adenosine metabolites in medital gastrocnemius muscles of mice have been described (Strick, 1995; Steeghs et al., 1996).

\textsuperscript{3}P-NMR Spectroscopy

In vivo \textsuperscript{3}P-NMR spectra of mouse hind-limb muscles were recorded on an Oxford Instruments magnet (4.3 T) equipped with a S.M.I.S.
spectrometer and working at 73 MHz. Probe characteristics and experimental conditions for the 31P-NMR spectroscopy at rest and during lower limb muscle contraction, as well as for inversion transfer, were essentially as described previously (Heerschap et al., 1988; van Deursen et al., 1984). Spectra in muscles at rest were acquired with a 5 s pulse interval (to avoid excessive saturation of PCR resonance in double-mutant muscle) in blocks of 48 scans each, giving a time resolution of 240 s per spectrum. After acquisition of a spectrum at rest, 2 spectra were obtained during 8 min of electrostimulation at 1 Hz (square pulses of 2 ms at 15 V). The muscles were allowed to recover for 8 min, during which 2 additional spectra were acquired, before another 8 min stimulation period of 5 Hz was applied. Subsequent muscle recovery was monitored for variable lengths of time. pH values were calculated from the chemical shift of the P3 signal.

Physiological Measurements of Skeletal Muscle Function
Contrastive characteristics and force measurements of medial gastrocnemius muscles were performed as described previously (de Haan et al., 1989). Muscle optimum length (L0) was first estimated using twitch contractions at different lengths of the muscle–tendon complex and further assessed using 3 tetani (duration, 150 ms; stimulation frequency, 100 Hz). Subsequently, the muscle performed 2 fatigue exercise protocols: one long duration (5 s) isometric tetanus at L0 and a series of 20 repeated isometric contractions (duration 170 ms) within 5 s at L0. Force signals were digitized (1000 Hz) and analyzed for peak force, time to peak force, and half time of relaxation (time for force to fall from half to a quarter at the end of stimulation; Edwards et al., 1975).

Electron Microscopy and Histochemical Fiber Typing
Mice were anesthetized intraperitoneally (using 2,2,2-t tribromoethanol) prior to the removal of the GPS muscle complex, heart, diaphragm muscle, and intercostal muscles. Sample preparation, pre- and postfixation, and Epon embedding were exactly as described (van Deursen et al., 1993). For light microscopic analysis, semithin 1 μm sections were cut and stained with toluidin blue following routine procedures. Subsequently, ultrathin sections were cut, double contrasted with uranyl acetate, and examined in a Philips electron microscope EM 301 or a JEOL TEM 1010. For routine histochemical examination, cross-sections of the GPS muscle groups were stained as described (Van Deursen et al., 1993).

[Ca2+] Measurements in Cultured Skeletal Muscle Cells
Hind-leg muscle from 10- to 25-day-old wild-type and homozygous mutant animals was dissociated, and the isolated satellite cells were grown on collagen-coated glass coverslips (25 mm diameter) in serum containing media for 2 days (Benders et al., 1984). Cells were allowed to differentiate for 4 days, loaded with 10 μM Fura 2 for 1.5 hr, and single-cell fluorescence intensity ratios (340/380 nm) were collected using conventional video microscopy and converted to Ca2+ as previously described (Benders et al., 1994). For calibration, Ca2+-saturated or Ca2+-free dye was set by 2 μM Ionomycin in the presence of 1.8 mM Ca2+ (pH 7.5) or 20 mM EGTA (pH 8.0), respectively. Under these conditions, the myotubes remained attached to the coverslip. The calibration parameters did not differ between the wild-type, M-Ck++, and CK− muscle cells.

Statistics
The unpaired t test was used to compare between wild-type animals and each of the mutant groups. Significance was set at p < 0.01 or 0.05.

Acknowledgments
We thank Patricia Groenen [Department of Cell Biology and Histology, University of Nijmegen], Klaas Nicofay (NMR Bijvoet Centre for Biomolecular Research, Utrecht), Ron Wevers (Department of Neurology and Paediatrics, University of Nijmegen), and Nelson D. Goldberg [Department of Biochemistry, University of Minnesota, Minneapolis, MN] for help and helpful discussions, and Antoon Jansen, Chris Spink, Irene Jetten, Xavier Martins Dias, Carla Offringa, and Christy Niemeyer for technical assistance. This work was supported by a program grant from the Dutch Organization for Scientific Research (Medical Sciences) and by the Dutch Heart Association and the Prinses Beatrix fonds.

Received October 28, 1996; revised February 19, 1997.

References
regulates calcium leak from agonist-sensitive internal calcium stores. FASEB J. 10, 302-308.


