CREATINE KINASE DEFICIENCY IN STRIATED MOUSE MUSCLE

Biochemical and physiological studies

Creatine kinase deficiëntie in dwarsgestreepte muizenspier
Biochemische en fysiologische studies

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen

op dinsdag 27 mei 2003 des middags te 4.15 uur

door

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geboren op 20 mei 1975, te Groningen
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ISBN 90-393-338-6

The research described in this thesis was carried out at the Department of Experimental In Vivo NMR of the Image Sciences Institute, University Medical Center Utrecht, Utrecht, The Netherlands. The project was financially supported by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO, project number 700-30-028).
Aan Opa
### Abbreviations

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>ACR</td>
<td>acceptor control ratio</td>
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<tr>
<td>ANT</td>
<td>adenine nucleotide translocator</td>
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<tr>
<td>AP, A</td>
<td>P₁, P₅-di(adenosine-5’)-pentaphosphate</td>
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<tr>
<td>AT</td>
<td>atractyloside</td>
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<td>CK</td>
<td>creatine kinase</td>
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<td>Cr</td>
<td>creatine</td>
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<td>CS</td>
<td>citrate synthase</td>
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<td>EDL</td>
<td>extensor digitorum longus</td>
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<tr>
<td>FCCP</td>
<td>carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone</td>
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<tr>
<td>Glc</td>
<td>glucose</td>
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<tr>
<td>HK</td>
<td>yeast hexokinase</td>
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<tr>
<td>IMS</td>
<td>inter membrane space</td>
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<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
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<td>Mi-CK</td>
<td>sarcomeric mitochondrial CK</td>
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<td>M-CK</td>
<td>myofibrillar CK</td>
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<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
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<tr>
<td>OXPHOS</td>
<td>oxidative phosphorylation</td>
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<td>PCr</td>
<td>phosphocreatine</td>
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<td>RCR</td>
<td>respiratory control ratio</td>
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<td>SOL</td>
<td>soleus</td>
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<td>VDAC</td>
<td>voltage-dependent anion channel</td>
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Chapter 1

Introduction
Chapter 1
**INTRODUCTION**

**MUSCLE ANATOMY AND PHYSIOLOGY**

Muscles are organized such that they can perform their function in everyday life. Figure 1 shows a schematic representation of the major steps controlling muscle function. Starting with a neural input signal, a calcium signal switches on myosin ATPase activity, generating mechanical output. When the neural signal is halted, the calcium signal diminishes by the action of the Ca\(^{2+}\) ATPase, ending myosin ATPase activity and subsequent mechanical output. In this cascade the availability of ATP free energy to the myosin and cation pump ATPases is of crucial importance. In muscle, the delivery of this ATP free energy is mainly provided by the mitochondrial ATPase in the process of oxidative phosphorylation. Within the muscle cell ATP supply by the mitochondria must balance ATP demand by the extramitochondrial ATPases in order to enable sustained mechanical output.

Muscle force output is made up of a cascade of several steps within the muscle cell. The contractile units contain a regulatory system plus actin and myosin that form the sliding filaments. At resting Ca\(^{2+}\) levels, approx. 100 nM in slow- and fast-twitch fibers, the overlapping myosin and actin filaments do not develop force because only weak or no interactions exist between the myosin cross-bridges and actin filaments. However, the excitation-contraction-relaxation (E-C-R) cycle can be started by an action potential along the sarcolemma and transverse tubular system followed by communication through the dihydropyridine receptor and ryanodine receptor. This finally leads to opening of the Ca\(^{2+}\) release-channels in the sarcoplasmatic reticulum (SR). During this Ca\(^{2+}\) release, the inhibition of the calcium-dependent regulatory system is temporarily removed allowing cyclic interactions to occur between myosin cross-bridges and actin, fuelled by ATP. The ATP for this force development is provided by oxidative phosphorylation and glycolysis. An important factor in determining the cyclic contraction speed is the Ca\(^{2+}\) re-uptake rate of the sarcoplasmatic reticulum Ca\(^{2+}\) pumps [1]. This contraction speed is a determinant in categorizing muscle cells. So-called fast-twitch fibers can be subdivided into glycolytic IIa and oxidative Iib fibers, both with fast twitch-kinetics and relatively high fatigability. In comparison, type I fibers are slow, more fatigue resistant and are able to sustain prolonged stimulation, while displaying slower twitch-speeds and lower power output. The muscle phenotype is determined by ATP demand and supply factors like myosin heavy chain (MHC) composition determining the ATPase demand and enzymatic components of glycolysis (e.g. lactate dehydrogenase) and oxidative phosphorylation (e.g. respiratory complexes) determining supply. Finally, muscle phenotypes display a mixture of both slow and fast fibertypes that can, furthermore, undergo a shift in composition depending on external factors like neuromuscular activity and exercise training [2].
Chapter 1

MUSCLE ENERGY METABOLISM

Oxidative phosphorylation

Mitochondria harbor the machinery to derive energy from the aerobic breakdown of pyruvate and fatty acids. Figure 2 displays the major steps of oxidative phosphorylation in mitochondria. These organelles consist of an inner and outer membrane which define two aqueous compartments, the matrix and the inter membrane space. The inter membrane space is very narrow and forms a compartment between the inner and outer membrane. Similar to the mitochondrial matrix, the inter membrane space contains a high protein concentration [3].

In the first step of oxidative phosphorylation delivery of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH₂) reducing equivalents from β-oxidation, glycolysis, or citric acid (TCA) cycle to mitochondrial dehydrogenases within the electron transfer chain takes place. Next, protons are actively transported from the matrix to the inter membrane space (IMS) by the respiratory complexes I, III and IV. Oxygen is the final electron acceptor at the level of complex IV.

The actual synthesis of ATP takes place according to the chemiosmotic theory of Mitchell [4], whereby the electrochemical gradient of protons (Δp) generated across the inner mitochondrial membrane by the electron transfer chain is used to synthesize ATP. The F₁F₀ proton ATP synthase allows the pumped protons to return to the matrix through its F₀ subunit and simultaneously catalyzes the phosphorylation of ADP. Finally, the adenine nucleotide translocator (ANT) catalyzes the exchange of ATP against ADP, thereby transporting ATP to the IMS. Regulation of mitochondrial activity to match changes in free-energy demand is complex due to the many possible control sites [5].

**Figure 1** Schematic diagram of ATP free energy metabolism in skeletal muscle. The myosin ATPase (I), the cation pump ATPase (II), and the mitochondrial ATPase (III), jointly constitute the major components of the free-energy network of the muscle cell. Starting with a neural input signal, Ca²⁺ switches on the myosin ATPase leading to the generation of mechanical output. Removal of the Ca²⁺ signal is catalyzed by the action of the cation pump ATPase. The energy balance that is required for sustained mechanical output is achieved by the activity of the mitochondrial ATPase (III) (adapted from [46]).
Initial studies by Chance and Williams showed the rate of oxidative phosphorylation in isolated mitochondria to be dependent on the extramitochondrial ADP concentration [6]. This feedback control theory is based on direct control of mitochondrial activity by cellular ATP demand [7]. The importance of the adenine nucleotide translocator (ANT) as a site of high control over mitochondrial respiration was shown by [8]. However, fluctuations in cellular Ca\(^{2+}\) levels have also been demonstrated to play an important role in regulating mitochondrial activity by modulating matrix dehydrogenase and F\(_{1}\)F\(_{0}\)-ATPase activity [9, 10].

In heart it has been established *in vivo* that mitochondrial activity in heart is highly sensitive to Ca\(^{2+}\). ADP levels appear unchanged in heart muscle over a wide range of workloads [11]. However, regulation of heart mitochondrial activity cannot solely be explained by Ca\(^{2+}\) signaling [12]. Mitochondrial activity in skeletal muscle is known to be mainly regulated by cytosolic ADP [13].

**Glycolysis**

Anaerobic production of ATP takes place in the cytosol converting glucose, via pyruvate, into lactate with the concomitant production of ATP. During the first stage of glycolysis ATP is consumed, while in the second stage NADH and ATP are generated. The overall yield is two ATP and pyruvate molecules from one glucose molecule. The energy yield of the glycolysis is rather low compared to that of oxidative phosphorylation, which amounts to 18 ATP molecules per pyruvate molecule. Nevertheless, glycolysis is an important source of energy during bursts of muscle workload. Furthermore, glycolysis represents the main ATP supply for cation homeostasis [14] and is of prime importance under anoxic conditions. In skeletal muscle the large store of glycogen can be seen as energy back up through the glycogenolysis pathway [3].

**Role of creatine kinase in energy metabolism**

Creatine kinase (CK: EC 2.7.3.2) catalyzes the phosphorylation of creatine (Cr) using ATP:

\[
\text{Mg-ATP}^{2-} + \text{Cr} \leftrightarrow \text{Mg-ADP}^{-} + \text{PCr}^{2-} + \text{H}^{+}
\]

CK is a member of the guanidino kinases family, which shows strong structural similarities. In vertebrates four different CK genes are present resulting in five CK isoforms with distinct patterns of subcellular localization, tissue-specific expression and developmental profile. CK is both localized near the sites of energy supply (mitochondria and glycolysis) and near the sites of energy demand (myosin and cation-pump ATPases). The cytosolic CK isoforms are encoded by two nuclear genes resulting in three possible dimers: BB-CK, MB-CK and MM-CK (denoted ‘M-CK’ in this thesis), where B and M denote brain- and muscle-type, respectively.

The homodimer of cytosolic M-CK has a molecular weight of 80 to 86 kD and is predominantly expressed in skeletal muscle, where it is responsible for more than 95% of the CK activity. BB-CK is found in all tissues in the fetus but in striated muscles it is replaced by MM-CK upon maturation and only trace amounts of BB-CK remain present [15].
Together with cytosolic CK, two mitochondrial CK isoforms, sarcomeric (sc) and ubiquitous (ub) CK, are expressed in excitable tissues [15]. ScMi-CK is exclusively found in cardiomyocytes, slow oxidative and fast oxidative-glycolytic myocytes of heart and skeletal muscle, together with M-CK. UbMi-CK is only expressed with B-CK. Mi-CK is encoded by nuclear DNA and a 39 amino-acid N-terminal presequence ensures Mi-CK translocation from the cytosol into the IMS of the mitochondrion. Mi-CK primarily forms cubic octamers with a molecular mass of about 340 kD. *In vitro* studies have shown that octameric Mi-CK can form a multienzyme complex with the voltage dependent anion channel VDAC, or porin, of the mitochondrial outer membrane and the adenine nucleotide transporter (ANT) in the inner mitochondrial membrane. It has been hypothesized that this multi-enzyme complex could be present *in vivo* and would enable channeling of high energy phosphates via PCr from the mitochondrial matrix to the cytosol [15, 16].

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**Figure 2** Schematic representation of the major metabolic steps in muscle cells. Within the mitochondria β-oxidation and TCA cycle deliver reducing equivalents (NADH + FADH₂) to oxidative phosphorylation. Next, protons are actively transported from the matrix to the intermembrane space (IMS) by the respiratory complexes I, III and IV. Oxygen is the final electron acceptor at the level of complex IV. The FₐFₒ proton ATP synthase allows the pumped protons to return to the matrix through its Fₒ subunit and simultaneously catalyzes the phosphorylation of ADP. The exchange of ATP against ADP is catalyzed by adenine nucleotide translocator (ANT), located in the inner membrane (IM). Finally, the VDAC, the pore protein in the outer membrane (OM), allows permeation of metabolites, including ATP, ADP, PCr and Cr. Creatine kinase is located in the IMS (Mi-CK) and in the cytosol (M-CK). Mitochondria and glycolysis in the cytosol are the sites of energy production. The sites of energy demand are the myosin and cation pump ATPases. During workload transients, the large PCr pool in the cell in combination with high CK activity can temporarily buffer ATP levels. In addition to direct ADP and ATP transfer, CK can communicate changes in ATP/ADP ratio from the cytosol to Mi-CK in the IMS via changes in PCr/Cr ratio.
Functional properties of creatine kinase

Since the discovery of the CK reaction in 1934 by Lohmann, who considered its importance for muscle contraction [17] it has generally been accepted that the CK system serves as a so-called ‘temporal energy buffer’ (Fig. 2). When the energy demand exceeds the ATP synthesis rate by glycolysis and/or oxidative phosphorylation CK/PCr can temporarily buffer the ATP/ADP ratio. The CK system has been proposed also to function as a ‘spatial energy buffer’. This function was originally suggested by Bessman et al. [18]. In this model PCr serves as an energy carrier. ATP synthesized by the oxidative phosphorylation in the mitochondrial matrix donates a phosphate group to Cr upon the formation of PCr and ADP. This reaction is catalyzed by Mi-CK. PCr then diffuses out of the mitochondrion to ATP hydrolyzing sites where ATP is regenerated from PCr. This reversed reaction is catalyzed by cytosolic CK and generates Cr that diffuses back to the mitochondrion to complete the energy shuttle [18].

Apart from the above free-energy transport and buffering functions the CK reaction keeps the ADP/ATP ratio high during high workloads [19]. A strong increase in the free ADP concentration would result in partial product inhibition of cellular ATPases, and a net loss of adenosine nucleotides through the reactions catalyzed by adenylyl kinase and AMP-deaminase [20]. Furthermore CK-mediated buffering of free energy homeostasis near the cellular sites of ATP supply and demand can provide optimal local ATP/ADP ratios that increase the thermodynamic efficiency of ATP hydrolysis as well as ATP synthesis. Cytosolic M-CK can maintain a high ATP/ADP ratio near the myosin and cation pump ATPases for an optimized ATP utilization. Furthermore, functional coupling of cytosolic CK with glycolytic ATP-delivering enzymes and of Mi-CK with the oxidative phosphorylation could keep local ATP/ADP ratio low, thermodynamically favoring ATP synthesis [15].

A similar model consists of coupling of PCr production to the oxidative phosphorylation by a ‘metabolic wave propagation’ [21, 22]. A decrease in the ATP/ADP ratio by hydrolysis of ATP at subcellular sites of high-energy phosphate utilization generates a local rise in the Cr concentration due to rapid hydrolysis of PCr via CK activity. A chain reaction is initiated in which the transfer of high energy phosphoryl groups takes place along a chain of equilibrated, soluble M-CK enzymes. Finally, Mi-CK presents the signal from these coupled reactions of ATP-Cr exchange to the mitochondria.

Furthermore, the CK system is involved in proton buffering. At the onset of metabolic work ATP is hydrolyzed to P_i, ADP and H^+ and a local accumulation of protons is avoided by the CK reaction. Another role of CK at the onset of muscle workload is the stimulation of glycolysis via the release of P_i from PCr hydrolysis.

Mathematical modeling of creatine kinase function

Quantitative mathematical modeling of CK function has provided insight in the role of CK in maintaining free energy homeostasis and control of respiratory flux. The first description of the energy cycle in muscle cells was presented by Chance and connected muscle workload to PCr content, emphasizing the buffer role of CK [23] (Fig. 3A). This model was recently expanded by the addition of adenylyl kinase activity, which represents an increase in the temporal ATP/ADP ratio buffer capacity [24]. In a model analogous to a simple electric circuit, Meyer modeled the creatine pool (coupled to high CK activity) as a biocapacitance in a linear model of the ATPase network in muscle [25] (Fig 3B). This capacitance will dampen the temporal signaling between the sites of
energy supply (mitochondria) and energy demand (myosin and cation pump ATPases), by using the PCr pool as buffer. In a more recent approach, Kushmerick released the CK local equilibrium constraint and predicted asymmetric dampening by CK at the onset and end of ATPase workload, leading to faster mitochondrial response at the onset of ATP demand [26].

An important factor in effectively describing muscle cell energetics is the compartmentation of the muscle cell. The importance of this spatial component was made explicit by Meyer [27] and illustrated the importance of spatial buffering by CK. From this modeling it was concluded by Sweeney that the temporal buffering function of CK is of great importance in relatively large muscle cells, e.g. fast-twitch muscles, allowing for lower mitochondrial density to support peaks in energy demand [28]. The spatial buffering component of CK function was concluded to be of more importance in slow-twitch muscle fibers, which are tuned to a greater duty cycle [28]. The importance of spatial buffering by CK for slow-twitch muscle was, furthermore, shown in a detailed

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**Figure 3** Rationale of mathematical models describing CK function. (A) Model approach connecting muscle workload to PCr content by high creatine kinase activity. ATP free energy demand of the ATPase residing in the myofibrils is matched by ATP supplied by oxidative phosphorylation and by the PCr pool (coupled to CK activity). Temporal buffering by CK is established by dampening fluctuations in ATP/ADP ratio (adapted from [23]). (B) Temporal buffering function of CK described as biocapacitance in a model analogous to an electric circuit. Voltage ($V_m$) represents the ATP free energy potential available in the mitochondria and $V_o$ the cytosolic free energy of ATP hydrolysis. The cytosolic current ($I_{cyto}$) represents the cytosolic ATPase activity and capacitor ($C_{CK}$) represents the stored energy charge of CK coupled to the PCr pool. The mitochondrial capacity equals resistance ($R_{mito}$) and the accompanying current ($I_{mito}$, not indicated) represents the oxidative phosphorylation rate (adapted from [25]).
mathematical model of the compartmentalized energy transfer system in the cardiac cell [29].

**BIOCHEMISTRY AND PHYSIOLOGY OF LOSS OF CREATINE KINASE FUNCTION**

*Creatine analogue feeding*

Creatine analogue feeding has been extensively studied, with β-guanidinopropionic acid (β-GPA) being most frequently used [30-33]. The analogues are taken up by cells of muscle, heart and brain, and are subsequently phosphorylated by CK. Most of the analogues, including β-GPA are poor substrates for CK [30]. Analogue feeding results in minor decreases in contraction speed, burst and endurance performance in mouse skeletal muscle [32]. However, alterations in myosin heavy and light chain take place to slower isoforms [2, 28, 31]. Although analogue feeding results in a substantial reduction of the flux through the CK reaction in skeletal muscle, this flux continues to greatly exceed the ATP turnover at rest [34]. Importantly, although alterations on the energy demand side are only minor, the analogues cause significant upregulation of ATP supply by a twofold increase in mitochondrial capacity [33, 35]. Although increased glycogen content was observed in fast-twitch muscles from β-GPA fed animals [30], less glycogen was used during stimulation, probably due to the build-up of inorganic phosphate [36]. On the basis of the above data, the functional importance of the CK system was questioned due to the small changes in muscle burst (minor decrease) and endurance (minor increase) performance [30, 36]. However, substantial CK activity remains upon creatine analogue feeding and the approach does not allow the differentiation between individual CK isoenzymes.

*Creatine kinase deficiency*

In the last decade, progress in the genetic manipulation of mice has enabled the generation of knock-out animals, which lack specific CK isoenzymes. This approach enables the study of the functional roles of individual CK enzymes.

In skeletal muscle over 95% of CK activity originates from the M isoform [15]. M-CK isoenzyme deficiency (M-CK−−) resulted in the complete elimination of the NMR-detectable flux through the CK system at rest [37]. Although the phenotypes of M-CK−− mice are mild (and the animals obviously viable), muscle performance is remarkably altered in M-CK−− muscle. Burst activity is strongly impaired and endurance performance is improved [37]. M-CK−− muscle, furthermore, displays increased mitochondrial capacity, higher glycogen content, expansion of the mitochondrial network and alterations in myosin heavy chain composition to slower isoforms [37].

Adaptations observed in Mi-CK deficient muscle are a significant increase of the flux through the adenylate kinase system and an increase in glycolytic activity [38]. However, phenotypic changes in Mi-CK−− skeletal muscle were not observed [39]. However, in Mi-CK−− heart muscle it was shown recently that Mi-CK is the isoenzyme primarily responsible for myocardial energy homeostasis during different workloads [40].

Interbreeding of the two single CK knock-out mice resulted in a mouse deficient in both MM-CK and Mi-CK (MiM-CK−−) while trace amounts of BB-CK remained [39]. MiM-CK−− muscle displays similar burst and endurance performance as observed in M-CK−− muscle [39] emphasizing the buffer role of cytosolic creatine
kinase. Furthermore, problems with the release and uptake of Ca\(^{2+}\) result in prolonged relaxation times of the muscles following contraction [39]. In CK-deficient myotubes the Ca\(^{2+}\) removal rate and the amount of Ca\(^{2+}\) released per transient were gradually reduced during electrical stimulation, further emphasizing the role of CK in muscle cell Ca\(^{2+}\) signaling [41]. Proteomics studies on CK-deficient muscles have, furthermore, shown upregulation of the mitochondrial cytochrome c oxidase, phosphate carrier, ANT, and VDAC proteins in gastrocnemius muscle and changes in the glycolytic enzyme pattern in soleus muscle [42]. Interestingly, using mass spectrometry fingerprinting no large scale changes in mitochondrial proteins could be observed in MiM-CK\(^{-/-}\) heart [43].

Liver does normally not contain CK but in recent studies the consequences of expressing Mi-CK and B-CK in livers of transgenic mice have been studied using phosphorous NMR. This so-called ‘knock-in’ technique caused the free ADP concentration to decrease depending on the catalytic activity of CK expressed in these livers [44]. Another elegant and novel technique makes use of microinjection of CK enzyme into CK-deficient muscle fibers. This approach largely restored the characteristics of Ca\(^{2+}\) handling and force mechanics of the CK-deficient skeletal muscle to those of the wild-type phenotype [45].
INTRODUCTION

OUTLINE OF THIS THESIS

The overall aim was to obtain a better understanding of the phenotype of the CK-deficient mouse muscle. To achieve this goal, the system was studied at different levels of complexity.

In chapter 2 we characterized the adaptations of mitochondrial function in slow- and fast-twitch striated muscle phenotypes from CK-deficient mouse mutants. Preparations of isolated mitochondria were used to avoid potential problems of limited metabolite diffusion inherent to the use of the permeabilized fiber technique.

With the use of existing and novel data as a basis for a mathematical modeling study, in chapter 3 we explored the functional role of Mi-CK in the free energy signaling between the cytosolic and the mitochondrial compartment. The specific question raised was if deletion of a fully functional CK system alters this signaling pathway.

In chapter 4 we address an experimental aspect of the permeabilized muscle fiber preparation and studied the problem of limited metabolite diffusion, not related to system physiology, but inherent to this preparation itself.

The final two chapters of this thesis present work that was conducted at a higher level of integration, i.e. the intact muscle cell.

In chapter 5 we measured mitochondrial function in isolated extensor digitorum longus and soleus muscle and tested the hypothesis that lack of CK leads to one order of magnitude faster mitochondrial response times. Guided by the novel data obtained in chapter 2 mitochondrial function was explored in detail in vivo. Specifically, we tested if the reported impaired free energy state leads to higher basal and active rates of respiration. These studies, furthermore, addressed two aspects of CK-function: its role as biocapacitance and its role in facilitated diffusion of high energy phosphates.

We studied mechanical force production in relation to CK deficiency in chapter 6. The observed contractile adaptations underlined reported observations of lack of burst performance and enhanced endurance performance upon loss of CK function. In an effort to better understand the mechanisms underlying these alterations in muscle performance, we tested if the documented remodeling of the contractile elements actually leads to alterations in contractile economy.

Finally, in chapter 7 we discuss the adaptation strategies available to the muscle cell in response to genetic modifications in its biological hardware.

REFERENCES

INTRODUCTION

Chapter 2

Mitochondrial Affinity for ADP is Twofold Lower in Creatine Kinase Knock-Out Muscles: Possible Role in Rescuing Cellular Energy Homeostasis

Frank ter Veld, Jeroen A.L. Jeneson and Klaas Nicolay

European Journal of Biochemistry, under revision
SUMMARY

Adaptations of the kinetic properties of mitochondria in striated muscle lacking cytosolic (M) and/or mitochondrial (Mi) creatine kinase (CK) isoforms in comparison to wild-type (WT) were investigated in vitro. Intact mitochondria were isolated from heart and gastrocnemius muscle of WT and single- and double CK-knock-out mice strains (cytosolic (MM-CK−/−), mitochondrial (Mi-CK−/−) and double knock-out (MiM-CK−/−), respectively). Maximal ADP-stimulated oxygen consumption flux (State 3 $V_{\text{max}}$; nmol O₂ · mg mitochondrial protein⁻¹ · min⁻¹) and ADP affinity ($K_{50}^{ADP}$; µM) were determined by respirometry. State 3 $V_{\text{max}}$ and $K_{50}^{ADP}$ of M-CK−/− and MiM-CK−/− gastrocnemius mitochondria were twofold higher than WT, but unchanged for Mi-CK−/−. For mutant cardiac mitochondria, only $K_{50}^{ADP}$ of mitochondria isolated from the MiM-CK−/− phenotype was different (i.e. twofold higher) than WT. The implications of these adaptations for striated muscle function were explored by constructing force-flow relations of skeletal muscle respiration. It was found that the identified shift in affinity towards higher ADP concentrations in MiM-CK−/− muscle genotypes may contribute to linear mitochondrial control of the reduced cytosolic ATP free energy potentials in these phenotypes.
Chapter 2

INTRODUCTION

Excitable mammalian cells contain high activities of creatine kinase (CK, EC 2.7.3.2), which catalyzes the reversible exchange of a phosphoryl group between phosphocreatine (PCr) and ATP. The tissue-specific CK enzymes are subcellularly compartmentalized and consist of three cytosolic dimers: BB-CK (brain- and smooth muscle-specific), MM-CK (muscle-specific) and MB-CK heterodimers. Furthermore, there is mitochondrial CK (Mi-CK) which is located in the intermembrane space of the mitochondrion and mainly consists of octamers in vivo [1]. Mi-CK and M-CK have been hypothesized to jointly form an energy transport network in which Cr and PCr function as diffusible intermediates between sites of ATP synthesis and utilization, thereby buffering fluctuations in the ATP free energy potential, i.e. the ATP/ADP concentration ratio [2, 3]. The roles of Mi-CK and M-CK in this CK/PCr shuttle model are to maintain a high local ADP/ATP concentration ratio near the adenine nucleotide translocase (ANT) by transphosphorylation of mitochondrially generated ATP to PCr and a high local ATP/ADP ratio near extramitochondrial ATPases, respectively [4].

Loss of CK function either by depletion of creatine via beta-guanidinopropionic acid feeding [5, 6] or by deletion of CK-isoforms in striated muscle weakens control of ATP/ADP concentration ratios in the cellular ATPase network [7-10]. Elevated ADP concentrations compared to WT have been measured at steady-states set by comparable cytosolic ATPase rates in Mi-CK knockout hearts [8, 10] and M-CK knockout fast-twitch gastrocnemius muscle [7, 9] compared to WT. In the latter muscle-type, this is the case both at rest as well as during contraction, in spite of phenotypic adaptations of the muscle at protein level. For example, a shift in the myosin composition of the myofibrils towards slower, energetically more efficient isoforms has been documented for fast-twitch muscle in response to CK deletion [11].

The adaptive response of mitochondrial function in CK-deficient muscle cells is less well documented. Deletion of CK function leads to increased citrate synthase (CS) activity in skeletal muscle and an increased V\text{max} of ADP-stimulated respiration in gastrocnemius skinned-fibers [12]. Here we investigated if the ADP concentration increase found in CK-deficient muscle is accompanied by a compensatory, adaptive shift in mitochondrial ADP affinity towards these higher ADP concentrations. We measured the ADP-stimulated V\text{max} of respiration and the affinity for ADP (K_{50}^{ADP}) in isolated mitochondria from two extreme striated muscle phenotypes: slow-twitch heart and fast-twitch gastrocnemius muscle.

EXPERIMENTAL

Animals

Adult wild-type C57BL/6 mice were used as controls. Cytosolic muscle-type CK-deficient mice (M-CK\textsuperscript{-/-}), sarcomeric mitochondrial CK -deficient mice (Mi-CK\textsuperscript{-/-}) and double knock-out mice, deficient both in cytosolic muscle-type and sarcomeric mitochondrial CK (MiM-CK\textsuperscript{-/-}), were generated in the laboratory of Dr B. Wieringa (Nijmegen University, the Netherlands) by gene-targeting as described previously [11, 13]. Offspring obtained in the breeding program was genotyped by PCR analysis on a regular basis. All experimental procedures were approved by the Committee on Animal Experiments of the University Medical Center Utrecht and complied with the principles
of good laboratory animal care.

*Biochemicals*

Percoll was obtained from Pharmacia Biotech (Uppsala, Sweden). Essentially fatty acid free BSA, lyophilized *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (NAD+ specific form) and lyophilized yeast HK (essentially salt free) were obtained from Sigma (St. Louis, U.S.A.). ATP and ADP were obtained from Roche Diagnostics (Mannheim, Germany). All other chemicals used were of the highest grade available and were obtained from regular commercial sources.

*Preparation of heart muscle mitochondria*

The isolation of mitochondria from mouse heart was based on the procedure by Cairns et al. [14], which represents a modification of the technique of Sims [15]. For each preparation, four mice were sedated with diethyl-ether and decapitated after which beating hearts were removed. The hearts (approx. 500 mg total wet-weight) were quickly placed in isolation medium (IM, containing 250 mM mannitol, 10 mM HEPES, 0.5 mM EGTA and 0.1% (w/v) BSA, pH 7.4; adjusted with KOH). Next, the ventricles were carefully freed of blood, minced intensively in 5 mL IM using scissors and homogenized in a 12 mL centrifuge tube by 5 strokes (up and down) using a loosely fitting teflon pestle rotating at 1000 rpm. Large cell debris and nuclei were pelleted by centrifugation for 5 minutes at 500 x g_{avg} in a Sorvall SS34 rotor. Mitochondria were pelleted by centrifuging the supernatant for 5 minutes at 10,000 x g_{avg} in the same rotor. The mitochondrial pellet was resuspended in 2 mL 12% (v/v) Percoll in IM, loaded on a discontinuous density gradient consisting of 3 mL 26% (v/v) Percoll and 4 mL 40% (v/v) Percoll in IM and centrifuged for 5 minutes at 31,000 x g_{avg} in a Sorvall SS34 rotor. Three major bands were obtained and the purified mitochondria were collected from the bottom band containing high-density mitochondria. Finally, the mitochondria were washed with IM as described above and resuspended in 200 µL IM at a mitochondrial protein concentration of approximately 12 mg/mL. The isolations typically took 45 minutes and were carried out at a temperature of 0 to 4 °C.

*Preparation of gastrocnemius muscle mitochondria*

The isolation of mitochondria from mouse gastrocnemius was essentially the same as the above described heart mitochondria isolation procedure, with some minor modifications. Four mice were sedated with diethyl-ether and decapitated after which hindleg gastrocnemius muscles were removed, placed in IM and freed of fat-tissue. The muscle tissue was minced intensively in IM using scissors and homogenized in a centrifuge tube by 5 strokes (up and down) using a loosely fitting teflon pestle rotating at 700 rpm. To obtain gastrocnemius mitochondria, again a discontinuous density gradient was used. The 26% (v/v) Percoll layer was replaced with a 20% (v/v) Percoll layer. Two major bands were obtained and the purified mitochondria were collected from the bottom band containing high-density mitochondria. Finally, the mitochondria were washed with IM as described above and resuspended in IM to a mitochondrial protein concentration of approximately 5 mg/mL.

*Protein determination*

The protein concentration of the mitochondrial preparation was determined by the BCA assay (Pierce). The BCA reagent was supplemented with 0.1% (w/v) sodium
dodecylsulfate. BSA was used as standard.

**Measurements of respiratory parameters**

The rates of oxygen consumption (nmol O$_2$ ⋅ mg mitochondrial protein$^{-1}$ ⋅ min$^{-1}$) were determined at 25 °C, using a high-resolution oxygraph (OROBOROS, Innsbruck, Austria) and 0.1 mg mitochondria in mitochondrial medium (containing 200 mM sucrose, 20 mM HEPES, 20 mM taurine, 10 mM KH$_2$PO$_4$, 3 mM MgCl$_2$, 0.5 mM EGTA and 0.1% BSA, pH 7.4; adjusted with KOH). The final volume of the oxygraph chamber was 2.0 mL. The oxygen solubility of air-saturated mitochondrial medium was taken to be 221 nmol O$_2$ ⋅ mL$^{-1}$ [16]. Substrates were 10 mM pyruvate plus 2 mM malate, or 10 mM succinate (in the presence of 10 µM rotenone). Respiratory assays were typically carried out in the following order. Endogenous respiration (State 2) was measured before the sub-maximal stimulation of oxidative phosphorylation using 0.1 mM ADP while maximal ADP stimulated respiration (State 3) was initiated by adding 0.25 mM ADP. After the resting state (State 4) had again been reached, 12.5 µM atractyloside was added to measure the rate of ANT-inhibited respiration. Finally, approximately 2 µM FCCP was titrated into the oxygraph chamber to induce maximally uncoupled respiration. The apparent K$_{50}$ values for ADP, i.e. the concentration of ADP needed to induce half maximal respiration in isolated mitochondria, were determined by measuring respiration at increasing [ADP] in mitochondrial medium containing 10 mM succinate, 10 µM rotenone, 20 mM glucose and 0.3 IU/mL yeast hexokinase (type VI), for depletion of mitochondrially formed ATP. The ADP concentration of stock solutions was determined enzymatically as described before [17]. To assess functional coupling of Mi-CK to oxidative phosphorylation, respiration was stimulated at increasing [ADP] in the presence of 25 mM creatine. To obtain the rate of ADP-stimulated respiration, the rates of respiration were corrected for ‘leak’ respiration based on a dynamic computer model of oxidative phosphorylation in muscle [18] according to [19].

**Spectrophotometric determination of enzyme activities**

Creatine kinase activity was measured at 25 °C on a Beckman DU65 spectrophotometer using coupled enzyme systems. Briefly, CK activity was assayed according to [20] in the forward direction in a medium containing 10 mM imidazole, 2 mM EDTA, 10 mM Mg-acetate, 2 mM ADP, 20 mM N-acetyl-cysteine, 20 mM glucose, 5 mM AMP, 1 mM NAD$^+$, 50 µM AP$_5$A, 25 mM PCr (pH 7.4, adjusted with acetic acid). Hexokinase and glucose-6-phosphate dehydrogenase were added at 3 IU/mL and 2 IU/mL, respectively. Pyruvate kinase and lactate dehydrogenase were both added at 4.5 IU/mL. Lactate dehydrogenase [21], citrate synthase [22] and aryl esterase [23] enzyme activities were measured at 37 °C and pH 7.4 according to published methods. The media used in the above assays were adjusted to 0.2% Triton X-100 to obtain maximal enzyme activities in muscle homogenates and mitochondrial fractions. Total ATPase activities in suspensions of intact mitochondria were measured as described previously [24]. Care was taken to avoid detergent contamination and no Triton X-100 was added.

**Data analysis and statistics**

Oxygraph data-analysis was performed with high-resolution respirometry software (OROBOROS DatLab 2.1; Innsbruck, Austria). Apparent K$_{50}$ values were calculated using non-linear regression (KaleidaGraph 3.0, Synergy Software, Reading, U.S.A.)
assuming second-order Hill kinetics [25]. Reported data are presented as arithmetic means ± SE. Statistical analyses were performed using a Student’s *t*-test. Differences between means were considered significant if *P*<0.05.

**RESULTS**

*Isolation of mouse heart and gastrocnemius mitochondria*

Percoll density gradient centrifugation was added as a final purification step to obtain a high quality mitochondrial preparation. Citrate synthase (CS) activity was increased in Percoll purified mitochondria when compared to the heart homogenate and the crude mitochondrial preparation, albeit not significantly (Table 1). Based on the activity of aryl esterase (AE) as a microsomal marker, 7% of the microsomal contamination remained in the final mitochondrial preparation (on protein basis) when compared to the homogenate (Table 1). The final mitochondrial suspension was furthermore greatly deprived of lactate dehydrogenase (LDH) activity, as a cytosolic marker. One of the most important quality criteria for the final mitochondrial preparation is the respiratory control ratio (RCR). The crude mitochondrial fraction had a low RCR (2.6 ± 0.3) and a relatively high ATPase activity (Table 1). Considerable levels of contaminating ATPases remained in the final mitochondrial sample during isolation of mouse heart mitochondria when conventional differential centrifugation protocols were used (Table 1). The reduction of the ATPase activity in the final heart mitochondrial preparation was accompanied by a considerably higher RCR of 11.2 ± 1.7, using pyruvate/malate as substrate (Table 1). Percoll density gradient centrifugation also strongly increased the RCR of the gastrocnemius mitochondrial preparation, i.e. from 1.9 ± 0.4 to 5.9 ± 0.5, using succinate as substrate (data not shown).

![Table 1](image)

**Creatine kinase activity**

Table 2 shows the specific activity of creatine kinase (CK) in mouse heart and gastrocnemius homogenates as well as in mitochondria isolated from these wild-type and CK-deficient mouse tissues. In agreement with the genotypes, the CK activities in mitochondria isolated from Mi-CK⁻/⁻ and MiM-CK⁻/⁻ mouse heart were negligible. Importantly, the data show that there is no significant change in Mi-CK activity in the case of M-CK deficiency.

The total CK activity was significantly lower in the heart homogenate of the three CK-
deficient mice compared to WT mice. In the gastrocnemius homogenate the total CK activities of WT and Mi-CK<sup>−/−</sup> were not significantly different, which is in line with the low Mi-CK content in glycolytic gastrocnemius muscle. Isolated gastrocnemius mitochondria from WT and M-CK<sup>−/−</sup> mice displayed a relatively low specific Mi-CK activity, compared to heart mitochondria. In preparations of mitochondria isolated from Mi-CK<sup>−/−</sup> gastrocnemius the relatively high CK activity, compared to mitochondria from MiM-CK<sup>−/−</sup> muscle, is probably due to contamination with M-CK.

<table>
<thead>
<tr>
<th></th>
<th>Creatine kinase activity (nmol ADP ⋅ mg protein&lt;sup&gt;−1&lt;/sup&gt; ⋅ min&lt;sup&gt;−1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
</tr>
<tr>
<td>WT (4)</td>
<td>9607 ± 1539</td>
</tr>
<tr>
<td>Mi-CK&lt;sup&gt;−/−&lt;/sup&gt; (4)</td>
<td>5152 ± 365 *</td>
</tr>
<tr>
<td>M-CK&lt;sup&gt;−/−&lt;/sup&gt; (4)</td>
<td>3332 ± 265 *</td>
</tr>
<tr>
<td>MiM-CK&lt;sup&gt;−/−&lt;/sup&gt; (4)</td>
<td>172 ± 14 *</td>
</tr>
<tr>
<td></td>
<td>Gastrocnemius Mitochondria</td>
</tr>
<tr>
<td>WT (6)</td>
<td>9768 ± 960</td>
</tr>
<tr>
<td>Mi-CK&lt;sup&gt;−/−&lt;/sup&gt; (4)</td>
<td>13159 ± 1106 *</td>
</tr>
<tr>
<td>M-CK&lt;sup&gt;−/−&lt;/sup&gt; (6)</td>
<td>351 ± 93 *</td>
</tr>
<tr>
<td>MiM-CK&lt;sup&gt;−/−&lt;/sup&gt; (4)</td>
<td>0 ± 32 *</td>
</tr>
</tbody>
</table>

Table 2 Creatine kinase activities in muscle homogenates and isolated mitochondria from wild-type and CK-deficient mice. *, P<0.05 compared to WT. Number of preparations is shown in parentheses. The values represent mean ± SE.

**V<sub>max</sub> of heart and gastrocnemius mitochondrial respiration**

The basic respiratory rates for maximal ADP stimulated (State 3), the atractyloside inhibited (AT-)State and the optimally uncoupled (FCCP-)State were essentially identical across the different types of cardiac mitochondria (Table 3A). The RCR values of cardiac mitochondria ranged from 3.7 ± 0.4 to 4.8 ± 0.1. Interestingly, respiratory rates in State 3, AT-State and FCCP-State were significantly higher in isolated gastrocnemius mitochondria from M-CK<sup>−/−</sup> and MiM-CK<sup>−/−</sup> mice, compared to WT (Table 3B). The respiratory rates of isolated gastrocnemius mitochondria from WT and Mi-CK-deficient mice were not significantly different. An ACR, and not RCR, was calculated from ADP titration experiments using State 3 and AT-State rates due to the limited amount of mitochondria obtained from gastrocnemius muscle. The ACR values showed no significant differences and ranged from 4.9 ± 0.3 to 6.0 ± 0.4 (Table 3B).

**K<sub>50</sub><sup>ADP</sup> of heart and gastrocnemius mitochondrial respiration**

In the presence of creatine, the concentration of ADP needed to induce half maximal respiration in isolated mitochondria, the apparent K<sub>50</sub> value for ADP (K<sub>50</sub><sup>ADP</sup>), was expectedly and significantly lowered from 21.3 ± 2.8 to 15.8 ± 1.6 µM and from 20.5 ± 1.7 to 14.5 ± 0.2 µM for mitochondria from WT and M-CK<sup>−/−</sup> myocardium, respectively (Table 3A). For heart mitochondria from Mi-CK<sup>−/−</sup> and MiM-CK<sup>−/−</sup> mice, these values, in the presence of creatine, were 21.0 ± 4.7 µM and 32.2 ± 4.2 µM, respectively and did not differ when creatine was omitted (Table 3A). As such, the K<sub>50</sub><sup>ADP</sup> in the presence of
creatine, representative of the conditions in vivo, of heart mitochondria was twofold higher for MiM-CK−/− mice (P<0.05) and tended to be higher (1.3-fold; not significant) for Mi-CK−/− mice compared to WT.

<table>
<thead>
<tr>
<th></th>
<th>Respiratory Rate (nmol O₂·mg mito protein⁻¹·min⁻¹)</th>
<th>RCR (ACR)</th>
<th>App. K₅₀ for ADP (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>AT-State</td>
<td>FCCP-State</td>
</tr>
<tr>
<td>(A) WT (4)</td>
<td>152.0 ± 15.9</td>
<td>27.0 ± 2.5</td>
<td>130.3 ± 14.1</td>
</tr>
<tr>
<td>Mi-CK−/− (4)</td>
<td>123.7 ± 19.9</td>
<td>23.3 ± 3.7</td>
<td>114.8 ± 23.1</td>
</tr>
<tr>
<td>M-CK−/− (4)</td>
<td>130.5 ± 20.3</td>
<td>22.0 ± 2.8</td>
<td>119.0 ± 22.8</td>
</tr>
<tr>
<td>MiM-CK−/− (4)</td>
<td>126.7 ± 14.5</td>
<td>20.9 ± 1.6</td>
<td>112.7 ± 13.5</td>
</tr>
<tr>
<td>(B) WT (6)</td>
<td>45.5 ± 5.2</td>
<td>8.3 ± 1.5</td>
<td>43.0 ± 5.0</td>
</tr>
<tr>
<td>Mi-CK−/− (6)</td>
<td>37.0 ± 2.3</td>
<td>6.4 ± 0.7</td>
<td>34.0 ± 3.6</td>
</tr>
<tr>
<td>M-CK−/− (6)</td>
<td>80.1 ± 4.0 *</td>
<td>15.9 ± 1.7 *</td>
<td>75.2 ± 3.7 *</td>
</tr>
<tr>
<td>MiM-CK−/− (6)</td>
<td>82.4 ± 8.2 *</td>
<td>19.7 ± 1.9 *</td>
<td>92.6 ± 4.8 *</td>
</tr>
</tbody>
</table>

Table 3: Kinetic characterization of succinate/rotenone dependent respiration of isolated heart and gastrocnemius mitochondria from wild-type and CK-deficient mice. Respiratory rates of isolated mouse heart (A) and gastrocnemius (B) mitochondria (0.1 mg·mL⁻¹) were measured in mitochondrial medium (see Materials and Methods) containing succinate as substrate and rotenone. The RCR value (for A) is the respiratory control ratio, i.e. the ratio of State 3 over State 4 (not shown). The ACR value (for B) is the acceptor control ratio, i.e. the ratio of State 3 over the atractyloside-inhibited state. For the determination of apparent K₅₀ steady-state respiratory rates were measured at increasing [ADP]. Mi-CK activity was induced by adding 25 mM creatine (Cr). *, P<0.05 compared to WT. #, P<0.05 compared to minus creatine (-Cr). Number of experiments is shown in parentheses.

The apparent K₅₀ for ADP of gastrocnemius muscle mitochondria, in absence of creatine, were 7.0 ± 1.0 µM and 7.3 ± 1.0 µM for M-CK−/− and MiM-CK−/−, respectively, versus 2.4 ± 0.3 µM for WT, and 6.4 ± 0.8 µM and 5.7 ± 0.7 µM versus 3.5 ± 0.3 µM, respectively, when creatine was present (Table 3B). No differences were found between WT and Mi-CK−/− gastrocnemius mitochondria (Table 3B). K₅₀ for MI-CK−/− mitochondria from MiM-CK−/− was in all cases lower than for cardiac mitochondria (Table 3B). In addition, the sensitivity of WT mitochondria to the presence of creatine in the medium differed between gastrocnemius and cardiac preparations: addition of creatine to the medium significantly increased K₅₀ for gastrocnemius mitochondria by 40% (Table 3B). In contrast, the K₅₀ for gastrocnemius mitochondria from M-CK−/− and MiM-CK−/− mice was not sensitive to the presence of creatine in the medium, and was significantly higher than WT in both conditions studied.

DISCUSSION

In this study we compared the functional kinetic characteristics of mitochondria from wild-type and creatine kinase-deficient mice in fast-twitch gastrocnemius and slow-
twitch heart muscle, which represent two very different striated muscle phenotypes.

Fast-twitch glycolytic skeletal muscle
The main finding of our studies on mitochondria isolated from various CK genotypes of fast-twitch gastrocnemius muscles was a twofold higher rate of endogenous and State 3 respiration ($V_{max}$) and a twofold higher apparent $K_{50}$ for ADP for M-CK$^{-/-}$ and MiM-CK$^{-/-}$ mice compared to WT mitochondria (Table 3B). Mitochondria from Mi-CK$^{-/-}$ gastrocnemius had essentially the same respiratory properties as WT mitochondria (Table 3B). The finding of an adaptive increase in respiratory $V_{max}$ in M-CK$^{-/-}$ and MiM-CK$^{-/-}$ gastrocnemius mitochondria is in line with the results of previous studies on muscle homogenate that reported an increase of mitochondrial protein in these genotypes [11, 13, 26, 27]. Also, the results of polarographic measurements of respiratory $V_{max}$ (but not $K_{50}$ for ADP; see [28, 29]) in permeabilized M-CK$^{-/-}$ gastrocnemius fibers, which can be compared to our results in a straightforward manner, are similar [12]. Our present investigations did not provide insight into the exact sites of $V_{max}$ upregulation in M-CK$^{-/-}$ and MiM-CK$^{-/-}$ phenotypes in terms of activities of individual components of the respiratory chain. However, the impaired calcium homeostasis documented in fast-twitch muscle of cytosolic CK-mutant mice [30] may well have played a role in directing the increase in mitochondrial capacity via the recently discovered calmodulin-kinase calcium-signaling pathway controlling mitochondrial biogenesis [31].

The $K_{50}$ for ADP in the presence of creatine, representative of the conditions in living muscle, was 6.4 and 5.7 µM for M-CK$^{-/-}$ and MiM-CK$^{-/-}$, respectively, compared to 3.5 µM for WT gastrocnemius mitochondria. This twofold-decrease in affinity for ADP in these two phenotypes is physiologically relevant in view of the reported twofold higher ADP concentration in resting MiM-CK$^{-/-}$ hindleg muscles [9] as will be discussed below. The apparent $K_{50}$ for ADP is determined by the permeability of the outer mitochondrial membrane to ADP via VDAC porins [17] and the affinities of ANT and F1-ATPase for ADP [32]. The latter also introduces a dependence on the mitochondrial membrane potential and thereby on the respiratory substrate [33]. Interestingly, recent experimental data reveal a relative decrease in VDAC mRNA and protein expression compared to the expression of other mitochondrial proteins in M-CK$^{-/-}$ and MiM-CK$^{-/-}$ gastrocnemius muscle [26, 34] suggesting a lower permeability of OMM for adenine nucleotides. The decrease in ADP affinity of isolated M-CK$^{-/-}$ and MiM-CK$^{-/-}$ muscle mitochondria we found is therefore in line with these findings at the protein level.

Slow-twitch oxidative cardiac muscle
No significant differences in mitochondrial respiratory $V_{max}$ were found when comparing isolated mitochondria from heart muscle from Mi-CK$^{-/-}$, M-CK$^{-/-}$ and MiM-CK$^{-/-}$ mice with heart mitochondria from WT mice (see Table 3A). These findings are in line with previous studies on skinned ventricular fibers from Mi-CK$^{-/-}$ and M-CK$^{-/-}$ mice that reported no difference in respiratory $V_{max}$ compared to wild-type [12, 35]. Our finding of twofold higher $K_{50}$ for ADP of MiM-CK$^{-/-}$ heart mitochondria and the trend towards a higher $K_{50}$ for ADP in the case of Mi-CK$^{-/-}$ mitochondria correlates well with recent studies on perfused hearts from CK-mutant animals. In these studies a compromised capacity for free energy homeostasis was demonstrated in isolated perfused heart from Mi-CK$^{-/-}$ and MiM-CK$^{-/-}$ mice [8, 10], but not M-CK$^{-/-}$ mice [10, 36].
In this section we discuss the implications of the identified \( V_{\text{max}} \) and \( K_{50}^{\text{ADP}} \) adaptations of mitochondria with respect to the function of the integrated ATPase network of the active striated muscle cell in which specific CK isoforms are absent. When analyzing the kinetics of such a network in the living cell that is characterized by the presence of net flux, it is relevant to consider concentrations as response variables subject to flux balance in the network [37]. This is captured in Figure 1 showing the variation of the extramitochondrial ATP/ADP free energy potential [38]. This variation is quasi-linear over the full empirical operational flux domain of mitochondria in skeletal muscle (5-85% of \( V_{\text{max}} \) [25]) indicating robust mitochondrial control of the cytosolic potential [38, 39]. At respiratory rates above the maximal operational rate, control of this potential deteriorates resulting in a rapid drop of ATP/ADP in response to any additional mitochondrial workload increment without activation of anaerobic glycolysis.

The kinetic graph format of Figure 1 will now be used to illustrate qualitatively (i.e. focusing on trends rather than absolute numbers) the implications of the \( V_{\text{max}} \) and \( K_{50}^{\text{ADP}} \) adaptations of mitochondria for the physiology of (Mi)M-CK-deficient skeletal muscle. In order to do so, we first translated the relative change in \( K_{50}^{\text{ADP}} \) to \textit{in vivo} conditions on basis of literature. This was necessary because \( K_{50}^{\text{ADP}} \) values for isolated mitochondria are typically lower than estimated \textit{in vivo} values (5 \( \mu \)M (this study) versus 23-44 \( \mu \)M [25, 39, 40], respectively, for skeletal muscle, and 20-30 \( \mu \)M [41, 42] versus 80 \( \mu \)M [43], respectively, in cardiac muscle oxidizing glucose). For skeletal muscle, we thus obtained an \textit{in vivo} \( K_{50}^{\text{ADP}} \) of 72 \( \mu \)M for MiM-CK-deficient skeletal muscle on basis of an \textit{in vivo} \( K_{50}^{\text{ADP}} \) value for WT skeletal muscle of 44 \( \mu \)M [25] and the 1.6-fold increase in \textit{in vitro} \( K_{50}^{\text{ADP}} \) for MiM-CK-/- compared to WT (Table 3B). These translated \( K_{50}^{\text{ADP}} \) values together with measured \textit{in vitro} mitochondrial \( V_{\text{max}} \) rates were first converted to muscle \( V_{\text{max}} \) rates assuming 10.3 mg mitochondrial protein per gram of skeletal muscle tissue mass [44] and then used to construct flow-force relations for three cases: (I) WT muscle characterized by \( V_{\text{max}} = (V_{\text{max}})_{\text{WT}} \) and \( K_{50}^{\text{ADP}} = (K_{50}^{\text{ADP}})_{\text{WT}} \); (II) MiM-CK-/- characterized by \( V_{\text{max}} = 2*(V_{\text{max}})_{\text{WT}} \) and \( K_{50}^{\text{ADP}} = 2*(K_{50}^{\text{ADP}})_{\text{WT}} \); (III) a hypothetical case characterized by \( V_{\text{max}} = 2*(V_{\text{max}})_{\text{WT}} \) and \( K_{50}^{\text{ADP}} = (K_{50}^{\text{ADP}})_{\text{WT}} \) (Fig. 1).

In the final step, we calculated the ATP/ADP free energy potential in resting WT and MiM-CK deficient fast-twitch mouse EDL muscle on basis of reported PCr, Cr and ATP concentrations at 20\(^\circ\) C [45] and a value of 166 for CK-K\textsubscript{eq} [46] yielding ATP/ADP ratios of 533 and 163 for WT and MiM-CK-/- EDL, respectively. This approach was valid because at rest thermodynamic equilibrium is also established in MiM-CK-/- due to the presence of some remaining CK activity. The free energy offset-points of the ATPase network for the two genotypes are indicated in Figure 1 by dashed-lines. Clearly, control of the cytosolic ATP free energy potential in MiM-CK-/- fast-twitch muscle is compromised already under conditions of basal ATP-demand.

The flow-force relation for WT muscle first of all shows that without any adaptation of \( V_{\text{max}} \) or \( K_{50}^{\text{ADP}} \), mitochondria in MiM-CK-deficient skeletal muscle would only be able to match a small range of ATPase load increments above basal rate due to the shifted offset-point of the network from 10 to 60% of WT \( V_{\text{max}} \) (Fig. 1). This would pose a problem, since the absolute ATPase load during contraction in MiM-CK-deficient
Chapter 2

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Muscle is higher than for WT because of an increased basal rate associated with compromised Ca\(^{2+}\) homeostasis [30]. We obtained experimental proof for higher absolute respiration rates in MiM-CK-deficient mouse EDL muscles at one and the same contraction frequency compared to WT due to a significantly increased basal respiration rate (Chapter 5). Secondly, the relation for MiM-CK-deficient skeletal muscle (case II) shows that the adaptations of the kinetic properties of mitochondria in this genotype, as identified in the present study, rescue both absolute capacity to generate oxidative ATP supply flux as well as robust control of the cytosolic ATP free energy potential during workload increments. Case III illustrates that while the increase of \(V_{\text{max}}\) is essential to restore capacity for oxidative ATP synthase flux, the increase of \(K_50\) in MiM-CK-deficiency is crucial to retain also the second aspect of mitochondrial function, i.e. control of the cytosolic ATP free energy potential. In this light, it is of interest that a doubling of \(K_50\) has also been found in skeletal muscle of patients with mitochondrial

Figure 1 Qualitative illustration of flow-force relations in fast-twitch skeletal muscle of WT and MiM-CK-deficient mice. Extramitochondrial ATP free energy potential represented by the ATP/ADP ratio in skeletal muscle from WT and MiM-CK\(^{-}\) mice is plotted against muscle respiratory flux (\(JO_2\) in nmoles O\(_2\) \( \cdot \) g muscle\(^{-1}\) \( \cdot \) min\(^{-1}\)), based on converted mitochondrial respiratory \(V_{\text{max}}\) rates and extrapolated \(K_50\) values from table 3B (in the presence of creatine). Three cases are presented: (I) WT with \(V_{\text{max}} = (V_{\text{max}})_{\text{WT}}\) and \(K_50 = (K_50)_{\text{WT}}\); (II) MiM-CK\(^{-}\) with \(V_{\text{max}} = 2*(V_{\text{max}})_{\text{WT}}\) and \(K_50 = 2*(K_50)_{\text{WT}}\) and (III) a hypothetical case with \(V_{\text{max}} = 2*(V_{\text{max}})_{\text{WT}}\) and \(K_50 = (K_50)_{\text{WT}}\). The free energy ATP/ADP offset-points at rest of the ATPase network for the two genotypes (case I and II) are indicated by dashed-lines. The operational flux domains to balance ATPase demand within the ATPase network (5-85% of wild-type \(V_{\text{max}}\)) are indicated by thickened-line-sections and matching bars, showing robust mitochondrial quasi-linear control of the cytosolic free energy potential. The dashed bar indicates the remaining WT operational flux domain when free energy potential is compromised.
lesions reducing $V_{\text{max}}$ by 50% [47, 48]. In spite of the severely reduced capacity to generate ATP synthase flux, these muscles have residual capacity for contractile work accompanied by linear changes in cytosolic ATP free energy at low ATP/ADP potentials [47, 48].

Analogously, we can now explain the benefit of the increased mitochondrial $K_{50}^{\text{ADP}}$ in Mi-CK$^{-/-}$ and MiM-CK$^{-/-}$ hearts in which mitochondrial control of the cytosolic ATP free energy potential is compromised [10, 36]. One would perhaps have expected also a higher mitochondrial $V_{\text{max}}$ in these cardiac muscle genotypes. An attractive, but speculative, explanation for the lack of any such $V_{\text{max}}$ increase is offered by Lindstedt et al. [49] who have proposed that the volume ratio of mitochondria, sarcoplasmic reticulum and myofibrils in a striated muscle cell is optimized for the particular mechanical task of the muscle. Our results suggest that cardiac muscle may well be limited in its ability to increase mitochondrial volume without compromising mechanical function, at least in comparison to fast-twitch skeletal muscle.

In conclusion, we propose that an increase in oxidative capacity and a reduction of the ADP affinity both constitute adaptations of mitochondrial function to alleviate compromised temporal and spatial buffering of the ATP free energy potential due to specific CK deletions. A specific mechanism for the regulation of mitochondrial capacity has recently been identified [31]. It remains to be determined which regulatory mechanisms are involved in setting the apparent mitochondrial $K_{50}^{\text{ADP}}$.

Acknowledgements
We want to acknowledge Drs. F.N. Gellerich, E. Gnaiger, B. de Kruijff and B. Wieringa for expert advice. The authors furthermore wish to acknowledge Drs. B. Wieringa, F. Oerlemans and K. Steeghs (Nijmegen University) for supplying the transgenic mice. This research has been financially supported by The Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO).

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Chapter 3

Cytosolic Creatine Kinase Deficiency In Mouse Heart. Evidence For An Adaptive Increase In Outer Mitochondrial Membrane Permeability

Frank ter Veld, Frank J. Bruggeman, Jeroen A.L. Jeneson, Frank N. Gellerich and Klaas Nicolay

Adapted from: Molecular Biology Reports (2002) 29, 183-6
SUMMARY

In this study we focused on possible alterations in the compartmentation of mitochondrial creatine kinase-derived ADP, which may arise due to the absence of a fully functional creatine kinase network in mouse heart. Reconstituted systems in which mitochondria compete with pyruvate kinase for creatine kinase- or hexokinase-generated ADP revealed a significantly increased ability of external pyruvate kinase to scavenge ADP generated in the inter membrane space. Respiration decreased to 45% and 36% of ADP-stimulated respiratory $V_{\text{max}}$ at excess pyruvate kinase activity in suspensions of heart mitochondria from wild-type and cytosolic creatine kinase-deficient mice, respectively. The use of a two-compartment kinetic model to explain the above data suggests that the deficiency in cytosolic creatine kinase results in a 50% increase in outer mitochondrial membrane permeability.
INTRODUCTION

Excitable cells contain high activities of creatine kinase (CK), which catalyses the reversible exchange of a phosphoryl group between phosphocreatine (PCr) and ATP. The compartmentalized CK isoenzymes in the cytosol (M-CK) and mitochondria (Mi-CK) proposedly function as an energy buffering and transport system to avoid large fluctuations in ATP/ADP ratios across a broad range of free-energy demands [1]. The role of cytosolic CK is obvious, less clear is the role of Mi-CK. Mi-CK is thought to decrease the apparent K_M for ADP of oxidative phosphorylation (OXPHOS) and to maintain a high local ADP concentration near the adenine nucleotide translocator (ANT) [2]. Mitochondrial creatine kinase, located in the inter membrane space (IMS) of the mitochondrion, is compartmentalized with respect to the cytosol due to the restricted metabolite diffusion through the VDAC porins in the outer mitochondrial membrane (OMM) [2].

With the generation of transgenic mice that lack specific CK isoforms new possibilities arise to study Mi-CK function. Transgenic animals that lack M-CK show an increased skeletal muscle endurance performance, alterations in their mitochondrial content and ultrastructure, suggestive of adaptations at the mitochondrial level. Transgenic animals, which only lack Mi-CK, however, show no or less severe adaptations [3]. Studies with perfused hearts deficient in Mi-CK M-CK revealed almost normal heart function at moderate workloads, although the ATP synthesis rate of CK was only 9% of the OXPHOS ATP synthesis rate [4]. In permeabilized cardiac fibers deletion of M-CK leads to increased citrate synthase activity [5], again indicative of mitochondrial alterations.

In the present study reconstituted systems were used to investigate possible alterations in OMM permeability and compartmentation of ADP in the IMS of heart mitochondria from WT and M-CK deficient mouse heart. The systems consisted of purified intact heart mitochondria, endogenous CK or exogenously added hexokinase as ADP regenerating enzymes and a pyruvate kinase (PK)-based ADP scavenging system. The rate of oxidative phosphorylation, which competes for kinase generated-ADP and is an indicator of IMS [ADP], was measured as a function of PK activity [6].

In this study we focused on possible alterations in compartmentation of ADP, generated by Mi-CK, which we hypothesized to arise from to the absence of a fully functional CK network. The results obtained using the reconstituted CK/PK system revealed a significantly increased ability of PK to scavenge IMS ADP in mitochondria from M-CK^- heart. To estimate the extent of the possible alterations in outer mitochondrial membrane permeability that might be responsible for these changes, we used a two-compartment kinetic model to calculate diffusion rate constants.

MATERIALS AND METHODS

Heart mitochondria were isolated using a discontinuous Percoll density gradient. M-CK-deficient mice were generated by gene-targeting [3]. Controls were WT C57BL/6 mice. Respiratory rates were determined at 25 °C, using a high-resolution oxygraph (OROBOROS, Innsbruck, Austria) and 0.1 mg mouse heart mitochondria in sucrose medium (200 mM sucrose, 20 mM HEPES, 20 mM taurine, 10 mM KH_2PO_4, 10 mM succinate, 3 mM MgCl_2, 0.5 mM EGTA, 10 μM rotenone and 0.1% BSA; pH 7.4).
Respiration was stimulated by adding 2 mM ATP and 25 mM creatine or by adding 2 mM ATP and 20 mM glucose in the presence of approx. 0.75 IU/mg yeast hexokinase (HK), equal to the endogenous Mi-CK $V_{\text{max}}$ ADP generating flux. Excess, 250 U/mg, pyruvate kinase (PK) was titrated to scavenge ADP and, finally, atractyloside was added to block the ANT. Mitochondrial CK activities were measured in sucrose medium at 25 °C and pH 7.4 in the direction of ADP formation.

**RESULTS**

By studying the basal respiratory properties of isolated heart mitochondria from M-CK deficient mice it was observed that the respiratory capacity was not significantly different, compared to wild-type mice (Chapter 2). Furthermore, the sensitivity of respiration for [ADP], described in terms of its apparent $K_M$, showed no difference between heart mitochondria from M-CK−/− and wild-type heart (Chapter 2).

In Figure 1 the effect is shown of increasing PK activity on hexokinase- or creatine kinase-stimulated respiration of WT heart mitochondria. At increasing PK activity HK-induced respiration was completely suppressed, indicating that all ADP formed by HK was accessible to the extramitochondrial ADP scavenger (PK). When ADP was, however, synthesized by Mi-CK in the inter membrane space compartment respiration remained high at excess PK activity, compared to the HK system. With ADP being produced by Mi-CK within the IMS, the near State 3 respiration (leak-corrected using atractyloside) decreased from 443.1 ± 78.6 to 194.4 ± 18.5 nmol ATP · mg mitochondrial protein$^{-1}$ · min$^{-1}$ when an excess PK activity was added to respiring WT heart mitochondria (Table 1). For heart mitochondria from M-CK−/− mice the decrease in respiration was from 569.5 ± 62.5 to 203.7 ± 35.2 nmol ATP · mg mitochondrial protein$^{-1}$ · min$^{-1}$ at excess PK activity (Table 1). In WT heart mitochondria an excess PK decreased respiration to 44.7 ± 5.8% of State 3 (Table 1). In heart mitochondria from M-CK−/− mice, the excess PK activity was significantly better able to scavenge IMS ADP and only 35.6 ± 2.5% of State 3 respiration remained (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>no PK (State 3)</th>
<th>excess PK</th>
<th>excess PK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol ATP · mg mitochondrial protein$^{-1}$ · min$^{-1}$)</td>
<td>(%) State 3</td>
<td></td>
</tr>
<tr>
<td>wild-type (4)</td>
<td>443.1 ± 78.6</td>
<td>194.4 ± 18.5</td>
<td>44.7 ± 5.8%</td>
</tr>
<tr>
<td>M-CK−/−(4)</td>
<td>569.5 ± 62.5</td>
<td>203.7 ± 35.2</td>
<td>35.6 ± 2.5% *</td>
</tr>
</tbody>
</table>

Table 1 Mi-CK supported respiration at excess PK in mitochondria isolated from WT and M-CK−/− mouse heart. State 3 respiration (nmol ATP · mg mitochondrial protein$^{-1}$ · min$^{-1}$, P/O=2 for succinate supported respiration) was induced by endogenous ADP generating Mi-CK activity, next pyruvate kinase (PK) was titrated to excess activity to scavenge ADP. Remaining respiration (leak-corrected using atractyloside) at excess PK is expressed as % of State 3. * P<0.05 compared to WT. Number of preparations is shown in parentheses. The values represent mean ± SD.

Next, a two-compartment kinetic model of the experimental setup was designed (Fig. 2), which was based on $K_M$'s from literature and measured $V_{\text{max}}$'s in the PK titration...
The model was employed to test the hypothesis that the above observations on alterations in heart mitochondrial properties with CK deficiency, might be due to changes in OMM permeability. The kinetic model was indeed effective in describing the experimental steady-state fluxes (see Fig. 1) and allowed the estimation of the ATP and ADP gradients across the OMM at increasing PK activity. Using the kinetic model an estimate could be made of the ADP permeability coefficient, $K_p (\text{dm}^4 \cdot (\text{mg} \cdot \text{min})^{-1})$ (a product of the diffusion coefficient for ADP ($\text{dm}^2 \cdot \text{min}^{-1}$) and the pore area of VDAC per milligram mitochondrial protein ($\text{dm}^2 \cdot \text{mg}^{-1}$)) of OMM VDAC-porin needed to describe the PK titration experiments. At excess PK activity, the remaining respiration that competes for ADP produced by IMS Mi-CK, corresponded to estimated ADP permeability coefficients of 0.11 and 0.17 dm$^4 \cdot (\text{mg} \cdot \text{min})^{-1}$ for the OMM VDAC-porins of heart mitochondria from WT and M-CK$^{-/-}$, respectively.

Figure 1 Effect of increased PK activity on respiration of mitochondria isolated from wild-type heart. Respiration was stimulated by ADP that was generated by mitochondrial creatine kinase (dots) or extramitochondrial hexokinase activity (squares). The solid lines correspond to the best fits obtained from the two-compartment mathematical model (Fig. 2).
DISCUSSION

The modeling suggests that cytosolic CK deficiency results in an adaptive (circa 50%) increase in OMM permeability. Interestingly, experimental data on increased VDAC mRNA and protein expression are suggestive of an increased OMM permeability due to a higher copy number of VDAC pores in the OMM, rather than changes in the intrinsic permeation properties of the VDAC porin protein [7, 8]. However, it cannot be excluded that a reduction in the degree of functional coupling between Mi-CK and the ANT [1, 2] is at the basis of the observed adaptive response to M-CK deficiency.

Furthermore, unlike recent work of Gellerich et al. [6] in which flux-dependent concentration gradients across the OMM were quantified, the limited amount of isolated mitochondria obtained from mouse heart did, unfortunately, not allow for quenching and subsequent measurement of extramitochondrial [ADP]. Such experiments are needed to obtain direct evidence for changes in flux-dependent ADP concentration gradients in response to loss of M-CK function.
Acknowledgements

The authors wish to acknowledge Drs. B. Wieringa, F. Oerlemans and K. Steeghs (Nijmegen University) for supplying the mouse mutants. This research has been financially supported by The Council for Chemical Sciences of the Netherlands Organization for Scientific Research.

REFERENCES

MITOCHONDRIAL OUTER MEMBRANE IS NOT A MAJOR DIFFUSION BARRIER FOR ADP IN MOUSE HEART SKINNED FIBER BUNDLES

Olav Kongas, Marijke J. Wagner, Frank ter Veld, Klaas Nicolay, Johannes H. G. M. van Beek and Klaas Krab

Submitted for publication
SUMMARY

Oxygen consumption of mitochondria in saponin-skinned cardiac fiber bundles is known to be stimulated by ADP with an order of magnitude higher apparent $K_M$ to ADP than found for isolated mitochondria. Here we report that incubating skinned cardiac fiber bundles of wild-type or creatine kinase (CK) double-knockout mice with exogenous CK and creatine or with yeast hexokinase and glucose as ADP producing systems decreases the apparent $K_M$ of mitochondrial respiration in the bundles several fold. Because these externally added ADP regenerating systems exert their functional effects in the extramitochondrial compartment, this experimental result shows that the above high $K_M$ is mainly due to diffusion gradients outside the mitochondria and cannot for the greatest part be explained by the low permeability of the mitochondrial outer membrane. We conclude that the intrinsic affinity to ADP of mitochondria in mouse heart fiber bundles in situ is of the same order of magnitude as that of isolated mitochondria.
INTRODUCTION

ADP stimulates oxidative phosphorylation in isolated mitochondria with Michaelis-Menten type kinetics with $K_M = 10 - 20 \mu M$. Interestingly, it was found that in situ mitochondria in saponin-skinned cardiac fibers possess an order of magnitude higher apparent $K_M$ to ADP, $K_{app}^{ADP}$ than isolated mitochondria [1, 2]. It has been proposed that the high in situ $K_{app}^{ADP}$ is caused by a diffusion limitation for ADP across the mitochondrial outer membrane, and that isolation of mitochondria removes such a limitation [1]. An alternative proposal is that the diffusion limitation may reside outside the mitochondria [3].

The aim of the present study was to test whether the major diffusion limitation for ADP, causing the high $K_{app}^{ADP}$ in mouse heart skinned fiber bundles, resides in the mitochondrial outer membrane. The rationale was that incubating bundles with a soluble ADP producing system results in a redistribution of the ADP fluxes, revealing the location of the diffusion limitation (see Fig. 1). In this study we used the skinned cardiac fiber bundles of both wild-type (WT) and double CK knockout (KO) mice. The fibers were either supplemented with CK and creatine (Cr) or with yeast hexokinase (HK) and glucose (Glc) as two alternative ADP producing systems, neither of which crosses the mitochondrial outer membrane. Since both ADP producing systems were able to decrease the $K_{app}^{ADP}$ several fold, we conclude based on mathematical model analysis that in mouse heart, the affinity of in situ mitochondria is of the same order of magnitude as that of isolated mitochondria.

EXPERIMENTAL

Animals

Wild-type C57BL/6 mice (WT mice) and transgenic mice lacking both cytosolic muscle-type MM-CK and sarcomeric mitochondrial CK (Mi-CK) isoforms (KO mice) were used. The KO mice were produced at the University of Nijmegen, the Netherlands, as described previously [4]. The heart cells of the KO mice have a residual activity of the brain-type BB-CK isoform amounting to 5% of the total CK activity in WT mice [4]. However, most of this residual activity is removed from the fiber bundles during skinning.

Compared to the WT mice, the mitochondrial structure [4] and the protein abundance in mitochondria (except for Mi-CK and inactive aconitase precursor) [5] in cardiac cells of the KO mice seem to be unchanged.

Solutions

All solutions contained 2.77 mM CaK$_2$EGTA, 7.23 mM K$_2$H$_2$EGTA, 6.56 mM MgCl$_2$, 0.5 mM dithiothreitol, 20 mM imidazole. The calculated buffered free calcium concentration was 0.1 $\mu$M, a condition which prevents contraction of the bundles.

Solution A additionally contained 50 mM potassium 2-(N-morpholino)ethanesulfonate (K-MES), 5.3 mM Na$_2$ATP, 15 mM phosphocreatine and 50 $\mu$g/ml saponin. Solution B contained instead 100 mM K-MES, 3 mM K$_2$HPO$_4$, 5 mM glutamate, and 2 mM malate. The pH of all solutions was adjusted to 7.1 at 25 °C. Chemicals were obtained from Sigma or Roche.
Preparation
Skinned fiber bundles from WT and KO mouse hearts were prepared in solution A according to the method described earlier [3].

Respiration rate measurements
The skinned fiber bundles were incubated in an oxygraph in 1 ml solution B with 1 mg/ml of bovine serum albumin (BSA) added. In those cases where 100 IU/ml yeast-type IV HK or 300 IU/ml soluble CK (rabbit muscle, Sigma) were present, these enzymes were activated by adding 25 mM Glc or 50 mM Cr, respectively. Steady rates of oxygen uptake were recorded at various levels of added ADP during the linear phase [3]. Determinations were carried out at 25 °C using a high-resolution oxygraph (OROBOROS, Innsbruck, Austria). The medium was vigorously stirred. Medium equilibrated with air contained 215 µM oxygen.

Mathematical model
To analyze the K_{app}^{ADP} measurements, a reaction-diffusion model of a skinned fiber bundle was used as described earlier [3], reflecting the situation in Fig. 1. The model was supplemented with kinetics of MM-CK using Eqs. 7-9 from [6].

Statistics
Data are presented as means ± SE. The hypotheses were tested with ANOVA, followed by post hoc tests with Bonferroni’s corrections to compare the control group pairwise with all other groups. P<0.05 indicated significance.

RESULTS AND DISCUSSION
Table 1 shows the K_{app}^{ADP}’s for stimulation of oxygen consumption by ADP of the skinned cardiac fiber bundles from the WT and KO mice with and without additions of the components of ADP producing systems. The HK system (HK and Glc) decreased the K_{app}^{ADP} of both WT and KO bundles circa fourfold. This is in accordance with the observation made using rat heart skinned fiber bundles [3]. The strong effect of Cr on the K_{app}^{ADP} of the WT bundles, caused by endogenous Mi-CK activity, has been well documented [7, 8]. The new finding is that the exogenously added CK and Cr reduced the K_{app}^{ADP} of the KO bundles almost twofold. We did not observe a significant effect of the ADP producing systems on the maximal respiration rate of the bundles, which was 8.2 ± 0.6 µmol ⋅ g_{ww}^{-1} ⋅ min^{-1}.

Compared to the CK system, the HK system was more effective in reducing the K_{app}^{ADP} of the KO bundles. Simulations of these experiments with the reaction-diffusion model of a skinned fiber bundle predicted a reduction of the K_{app}^{ADP} from 114 µM to 74 and 45 µM for CK and HK systems, respectively (cf. Table 1), suggesting that the differential effect on the K_{app}^{ADP} that we measured experimentally was caused by the different activity and kinetics of HK vs. exogenous CK. In other words, the kinetic properties of HK make it a more effective ATP user in our experimental conditions than CK, despite the lower added HK activity. It is widely accepted that Mi-CK is very effective in reducing K_{app}^{ADP} because of its close proximity and presumable coupling to adenine nucleotide translocator. However, we found that exogenously added yeast HK is also very effective in reducing K_{app}^{ADP} in WT and KO fibers.
Table 1 Influence of various conditions on the respiration kinetics of skinned cardiac fiber bundles. Kinetics of stimulation of oxygen consumption by ADP in wild-type (WT) and creatine kinase knockout (KO) mouse heart bundles were studied with and without the following additions to the medium: Cr, 50 mM creatine; CK, 300 IU/ml creatine kinase (rate towards ATP production); Glc, 25 mM glucose; HK, 100 IU/ml yeast hexokinase (rate towards ADP production). $K_{\text{app}}^{ADP}$'s are given as means ± SE. Number of preparations is shown in parentheses. a, $P<0.001$ compared to control without additions; b, $P=0.0024$ and c, $P<0.001$ compared to KO without additions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$K_{\text{app}}^{ADP}$ ($\mu$M)</th>
</tr>
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<tbody>
<tr>
<td>WT (12)</td>
<td>226 ± 23</td>
</tr>
<tr>
<td>WT + Cr (4)</td>
<td>30 ± 3 $^a$</td>
</tr>
<tr>
<td>WT + CK (10)</td>
<td>259 ± 54</td>
</tr>
<tr>
<td>WT + CK + Cr (7)</td>
<td>25 ± 4 $^a$</td>
</tr>
<tr>
<td>WT + HK + Glc (5)</td>
<td>46 ± 6 $^a$</td>
</tr>
<tr>
<td>KO (5)</td>
<td>114 ± 13</td>
</tr>
<tr>
<td>KO + Cr (7)</td>
<td>107 ± 8</td>
</tr>
<tr>
<td>KO + CK (11)</td>
<td>109 ± 11</td>
</tr>
<tr>
<td>KO + CK + Cr (8)</td>
<td>61 ± 8 $^b$</td>
</tr>
<tr>
<td>KO + HK + Glc (5)</td>
<td>32 ± 4 $^c$</td>
</tr>
</tbody>
</table>

Thus Mi-CK is not necessary for the reduction of $K_{\text{app}}^{ADP}$, in accordance with the finding of Boehm et al. [8] (see below). Indeed, the reduction of $K_{\text{app}}^{ADP}$ is according to our diffusion model analysis well explained by ADP production by soluble HK or exogenously added CK distributed evenly inside the permeabilized fiber bundles, and not confined to the mitochondria. When interpreting the above results, it was assumed that neither HK nor CK significantly affect the mitochondrial outer membrane permeability. This assumption is supported by the following considerations. Many groups have failed to show the significant binding of yeast HK by mammalian or yeast mitochondria while, in contrast, those mitochondria bind the mammalian Type I HK avidly (for review see [9]). Though the yeast HK and mammalian Type I HK have similar amino acid sequences, the former lacks the hydrophobic NH$_2$-terminal segment that is critical for binding of the Type I isoenzyme [10-12]. Part of the MM-CK activity in muscle has been shown to be tightly coupled to ATPases in sarcoplasmic reticulum and plasma membrane (for review see [13]). However, we are not aware of direct evidence for association of MM-CK with mitochondrial membrane components. Based on the measurements by Veksler et al. [7], the in vivo soluble MM-CK activity in mouse heart cell was estimated to be about 420 IU/ml. Therefore it is unlikely that 300 IU/ml CK used in our experiments would involve such binding. Aspecific effects of CK and HK protein addition on the mitochondrial outer membrane permeability can be excluded, since incubation of the KO fibers with CK or either fiber-type with HK without the corresponding activating substrates had no effect on the measured $K_{\text{app}}^{ADP}$ (see Table 1 and ref. [3]).

Boehm et al. [8] observed a significant 45% decrease of the $K_{\text{app}}^{ADP}$ of the bundles from Mi-CK knockout mice as a result of Cr addition. They hypothesized that such a decrease would be due to coupling of the MM-CK to the VDAC pore in the outer mitochondrial membrane in these mice. In contrast, Kay et al. [14] found that adding Cr
caused only a non-significant 13% increase of the respiration rate of Mi-CK knockout bundles achieved with 1 mM ATP. Our model simulations of the latter experiment predict a 16% increase. Therefore, assuming high affinity of the mitochondria to ADP in these bundles resolves the seeming contradiction.

The methods used do not allow us to fully characterize the ADP diffusion path from medium to mitochondrial outer membrane. NMR measurements of the diffusion coefficients of various metabolites [15-17] lead to estimates of the ADP diffusion coefficient in the heart to be of circa 200 \( \mu m^2 \cdot s^{-1} \) at 25 °C [3]. To yield \( K_{app}^{ADP} \)'s of few hundreds of \( \mu M \) at such a diffusion coefficient, the effective diffusion distance for ADP should be more than 30 \( \mu m \), which has been observed in rat heart skinned fiber bundles [3]. However, this distance is several fold longer than the radius of cardiac cells (6 - 8 \( \mu m \)) that possess a \( K_{app}^{ADP} \) ranging from 150 [2] to 250 \( \mu M \) [18] after permeabilization.

To estimate the intracellular diffusion coefficient in these experiments, the contribution to diffusion resistance of unstirred layers and cellular aggregates in the cell suspensions should be quantified. Further studies are needed before the diffusion limitation between the major ADP producing sites and the mitochondria in the intact heart can be determined.

In summary, it was found that increasing the intrafiber ADP producing activity in two alternative ways decreased the \( K_{app}^{ADP} \) of the mouse heart skinned fiber bundles considerably. We conclude that the high \( K_{app}^{ADP} \) reflects mainly the diffusion gradients outside the mitochondria, indicating that the affinity of mitochondria to ADP inside the
skinned fiber bundles and perhaps also in the intact heart is of the same order of magnitude as that of isolated mitochondria.

Acknowledgments
This research was supported by a Marie Curie Fellowship of the European Community program Improving Human Research Potential and the Socio-economic Knowledge Base under contract number HPMF-CT-1999-00309 and by the Estonian Science Foundation's grant ETF4704. We are grateful to Drs. B. Wieringa, F. Oerlemans and E. van Gelderop who provided us with the CK knock-out animals. We thank Dr. S.P. Elmore for helpful discussions.

REFERENCES

Chapter 5

THE STATIONARY AND DYNAMIC STATES OF OXIDATIVE METABOLISM IN WILD-TYPE AND CREATINE KINASE-DEFICIENT MUSCLE

Frank ter Veld, Jeroen A.L. Jeneson and Klaas Nicolay

Submitted for publication
Chapter 5
RESPIRATION OF WT AND CK-DEFICIENT MUSCLE

SUMMARY

Quantitative mathematical models of CK function have partially predicted the network kinetics during stepwise changes in ATPase activity and the effects of CK deletion. According to these strictly temporal models, the kinetics of oxygen consumption at the onset of contractions in muscle lacking cytosolic CK activity should be dramatically faster compared to wild-type mice. Here we tested this prediction in isolated mouse muscle.

To that aim, extensor digitorum longus (EDL) and soleus (SOL) muscles were isolated from wild-type (WT) and knock-out mice deficient in the cytoplasmic muscle-type and sarcomeric mitochondrial isoenzymes of CK, and respiratory rates were measured at rest and during stimulation in intact muscle.

We observed a twofold increase in basal rate of respiration in both CK-deficient EDL and SOL muscle, which underlines the consequences of impaired energy homeostasis on the metabolic rate of the muscle cell at rest. The increase in mitochondrial content reported for CK-deficient fast-twitch muscle gave rise to higher respiratory rates in EDL muscle upon activation. Active respiration of CK-deficient SOL muscle was unchanged.

The loss of CK function in EDL and SOL muscle decreased the deactivation time of mitochondrial activity at the end of contractions, indicative of the lack of a temporal energy buffer. However, mitochondrial activation times in response to onset of contractions were found to be similar. This finding argues for preferred ADP signal transduction via creatine concentration changes in wild-type muscle cells communicated by high creatine kinase activities at the sites of energy supply and demand. Specifically, it appears that creatine kinase activity in the mitochondrial inter membrane space compartment is essential for fast mitochondrial response to workload transitions.
Chapter 5

INTRODUCTION

Creatine kinase (CK; E.C. 2.7.3.2) catalyzes the reversible transfer of a phosphate group from ATP to creatine [1]. The function of this reaction in excitable cells has been extensively studied, ranging from in vitro [2] and in vivo [3, 4] biochemical studies of CK kinetics and cellular activity distributions to physiological studies of striated muscles lacking CK activity [5-8]. The results have established that a activity of cytoplasmic-(M-CK) and mitochondrial CK (Mi-CK) in combination with a large creatine pool constitutes a functionally important temporal and spatial buffer of the cytosolic ATP/ADP ratio during step changes in cellular ATPase activity [1, 9]. Findings of impaired burst-activity and a rapid decline in peak force of contraction upon stimulation of skeletal muscle lacking M-CK have clearly illustrated this buffering function [8, 10].

This qualitative description of CK function in excitable cells was first made quantitatively explicit by Meyer [11]. The creatine pool coupled to high CK activity was modeled as a biocapacitance in a linear model of the ATPase network in muscle and as a source of facilitated diffusion for high-energy phosphates [3, 12, 13]. Based on these models the timecourse of phosphocreatine concentration changes during rest-work transitions for submaximal workloads should follow mono-exponential kinetics characterized by one and the same apparent time constant for activation and deactivation of contractile ATPase [13]. However, in a more recent model Kushmerick released the CK local equilibrium constraint and predicted activation kinetics of mitochondrial respiration to be faster at the onset of contractions, compared to deactivation kinetics [12]. Furthermore, it was predicted that the kinetics of oxygen consumption at the onset of contractions should be dramatically faster in muscle lacking cytosolic CK activity (one order of magnitude according to [12]) than in wild-type (WT) muscle [14]. The prediction of mono-exponential kinetics has been confirmed by 31P NMR spectroscopic measurements in mammalian muscle [13, 15]. The other two predictions have awaited rigorous testing. Moerland and Kushmerick [6] found only twofold faster rate constants for recovery of oxygen consumption following tetanic contractions in isolated hindlimb muscles from β-guanidinopropionic acid (β-GPA; an effective inhibitor of the CK reaction)-treated mice in comparison to WT. However, no data were obtained on the activation rate of respiration in these muscle phenotypes. Here, we tested the prediction that activation of muscle respiration at the onset of a step change in ATPase activity is faster than deactivation kinetics and we tested if CK ablation shortens the mitochondrial activation time by one order of magnitude.

EXPERIMENTAL

Animals and muscle preparation

Adult wild-type C57BL/6 mice were used as controls. Double knock-out mice, deficient in cytosolic muscle-type and sarcomeric mitochondrial CK (MiM-CK−/−), were generated in the laboratory of Dr B. Wieringa (Nijmegen University, The Netherlands) by gene-targeting as described previously [8, 16]. Offspring obtained in the breeding program was genotyped by PCR analysis on a regular basis. All experimental procedures were approved by the Committee on Animal Experiments of the University Medical Center Utrecht and complied with the principles of good laboratory animal care. Mice (age 21-30 days) were killed by cervical dislocation, EDL and SOL muscles of both hindlegs
were prepared free from the surrounding tissue and a 5.0 silk suture (Ethicon, Norderstedt, Germany) was attached to the muscle tendons.

**Simultaneous recording of muscle oxygen consumption and force development**

The rates of oxygen consumption (nmol O₂ g muscle weight⁻¹ min⁻¹) were determined at 20 °C, using a high-resolution oxygraph (OROBOROS, Innsbruck, Austria) with a stirring rate of 500 rpm. One end of the muscle was mounted in a custom-made oxygraph stopper and the other end was connected to an adjustable Harvard Apparatus 60-2995 force transducer (Harvard Instruments Limited, Edenbridge, UK), which allowed the muscle to be stretched to yield its maximum twitch force. Muscles were stimulated via platinum wire electrodes by supramaximal pulses (0.5 ms duration; 6-15 V) by a Grass S88 dual channel stimulator (Astro-med, West Warwick, RI, USA) and force signals were digitized at 1000 Hz sampling rate. The measurement protocol started with 10 minutes of rest to record basal respiratory flux followed by 5 minutes at 0.5, 1.0 or 2.0 Hz and 1.5 or 3.0 Hz for EDL and SOL muscles, respectively. Episodes of muscle stimulation were separated by 10 minute intervals during which non-stimulated basal respiration of the muscle was recorded.

Respiratory fluxes were corrected for chamber oxygen leak by measuring the exponential decay of P_{O2} in the oxygraph chamber at 20 °C containing Ringer solution (116 mM NaCl, 25.3 mM NaHCO₃, 4.6 mM KCl, 2.5 mM CaCl₂, 1.16 mM KH₂PO₄, 1.16 mM MgSO₄, pH of 7.4) according to [17]. Oxygen solubility of 5 % CO₂ – 95 % O₂ equilibrated Ringer medium was calculated according to [18] and oxygen electrode response times were constant at approx. 4 seconds (tested prior to each experiment). All measurements of muscle respiration were performed as randomized paired-experiments with simultaneous measurement of one WT and one MiM-CK⁻/⁻ muscle in a dual-chamber setup. To avoid measurements at high chamber oxygen-leak all experiments were started after 30 minutes of equilibration. To avoid oxygen limitation of respiration in SOL and EDL muscles at 20 °C [19] all measurements were performed above a P_{O2} of 450 Torr. Chamber volume (approx. 5.2 mL) and muscle weight (blotted and tendon free) were determined after each experiment.

**Data acquisition, analysis and statistics**

Oxygraph and force transducer data-acquisition was performed with LabView software (National Instruments, Woerden, The Netherlands). Absolute muscle respiratory rates and timeconstants were calculated using Origin 6.0 (Microcal Software Inc., Northhampton, MA, U.S.A.). Reported data are presented as arithmetic means ± SE. Statistical analyses were performed using a Student’s unpaired t-test. Differences between MiM-CK⁻/⁻ and WT muscle were considered significant if P<0.05.

**RESULTS**

**Mechanical performance**

EDL muscle weights were significantly lower for MiM-CK⁻/⁻ mice, compared to WT (Table 1). Weight of SOL muscles from MiM-CK⁻/⁻ mice was not significantly different compared to WT (Table 1).
Table 1 Physiological data for wild-type and CK-deficient (MiM-CK$^{-/-}$) muscles. Values are means ± SE. *, $P<0.05$ compared to WT.

<table>
<thead>
<tr>
<th></th>
<th>Mouse weight (g)</th>
<th>EDL weight (mg)</th>
<th>SOL weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>23.5 ± 0.6 (n = 16)</td>
<td>10.9 ± 0.4 (n = 7)</td>
<td>8.3 ± 0.2 (n = 6)</td>
</tr>
<tr>
<td>MiM-CK$^{-/-}$</td>
<td>24.9 ± 0.5 (n = 16)</td>
<td>6.1 ± 0.5 * (n = 6)</td>
<td>7.6 ± 0.4 (n = 6)</td>
</tr>
</tbody>
</table>

Initial twitch force per mg of muscle wet-weight was similar in MiM-CK$^{-/-}$ and WT EDL muscles. SOL muscles from MiM-CK$^{-/-}$ mice, however, showed a significantly lower initial twitch force (Table 2). During the first 20 sec of stimulation, at 1.5 and 1.0 Hz for EDL and SOL, respectively, no differences in fatigue were observed for MiM-CK$^{-/-}$ EDL and SOL muscles, compared to WT (Table 2). However, at 250 sec into the 1.5 Hz stimulation protocol, MiM-CK$^{-/-}$ EDL showed less fatigue compared to WT, 86.1 ± 3.2 % (n = 5) and 76.9 ± 2.3 % (n = 5) of initial force for MiM-CK$^{-/-}$ and WT EDL, respectively. The force decline at 250 sec of 1.0 Hz stimulation was similar for MiM-CK$^{-/-}$ and WT SOL muscle (Table 2).

Table 2 Development of twitch-force performance of wild-type and CK-deficient (MiM-CK$^{-/-}$) EDL and SOL muscles. EDL and SOL muscles were stimulated at 1 and 1.5 Hz, respectively. Values are means ± SE. *, $P<0.05$ compared to WT.

<table>
<thead>
<tr>
<th></th>
<th>Initial force (N/g)</th>
<th>After 20 s of stimulation (% initial force)</th>
<th>After 250 s of stimulation (% initial force)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EDL (n = 7)</td>
<td>3.4 ± 0.2</td>
<td>94.3 ± 1.8</td>
<td>76.9 ± 2.3</td>
</tr>
<tr>
<td>MiM-CK$^{-/-}$ EDL (n = 5)</td>
<td>2.9 ± 0.3</td>
<td>94.3 ± 3.3</td>
<td>86.1 ± 3.2 *</td>
</tr>
<tr>
<td>Wild-type SOL (n = 5)</td>
<td>2.9 ± 0.2</td>
<td>87.6 ± 2.5</td>
<td>71.4 ± 4.2</td>
</tr>
<tr>
<td>MiM-CK$^{-/-}$ SOL (n = 5)</td>
<td>2.1 ± 0.2 *</td>
<td>89.8 ± 0.9</td>
<td>74.6 ± 1.9</td>
</tr>
</tbody>
</table>

WT and MiM-CK$^{-/-}$ muscle respiration
Stationary states
Basal respiration rates of both EDL and SOL muscles from MiM-CK$^{-/-}$ mice were circa twofold higher compared to WT EDL and SOL muscles, respectively (Table 3 and 4). Initiation of contractions in EDL muscles resulted in a similar net increase in respiration in MiM-CK$^{-/-}$ and WT muscles at stimulation frequencies of 0.5, 1.0 and 2.0 Hz (Table 3). Net oxygen consumption rates (nmol O$_2$ · g muscle$^{-1}$ · min$^{-1}$) of MiM-CK$^{-/-}$ EDL muscles were therefore significantly higher at all stimulation frequencies studied (Table 3, Fig. 1A). Importantly, the relative increase of respiration for each doubling of EDL contractile duty cycle, scaled to the 0.5 Hz duty cycle, was significantly higher in MiM-CK$^{-/-}$ than WT muscle at 4 duty cycle equivalents (Fig. 1A, insert).
**Figure 1** Respiratory flux at increasing twitch-frequencies for wild-type (white bars) and CK-deficient (gray bars, MIM-CK⁻) EDL (A) and SOL (B) muscle. The insert in Figure 1A shows the respiration increase at increasing duty cycle (relative to 0.5 Hz). Values are means ± SE. *, P<0.05 compared to WT.
In SOL muscle, oxygen consumption rates at 1.5 and 3.0 Hz stimulation were similar in MiM-CK⁻ and WT muscles, even though basal respiration was twofold higher in the former (Table 4). Therefore, the oxygen consumption rate increase from basal to 1.5 Hz stimulation was significantly lower in MiM-CK⁻ SOL muscle (124.7 ± 12.1 nmol O₂ · g muscle¹ · min⁻¹ (n = 6)) compared to WT (183.6 ± 5.9 nmol O₂ · g muscle¹ · min⁻¹ (n = 6) (Table 4, Fig. 1B)). A similar trend, although not significant, was observed for MiM-CK⁻ SOL muscle stimulated at 3.0 Hz (Table 4, Fig. 1B).

### Table 3
Respiratory flux as a function of stimulation frequency for wild-type and CK-deficient (MiM-CK⁻) EDL muscle. Values are means ± SE. *, P<0.05 compared to WT.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type EDL</th>
<th>MiM-CK⁻ EDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol O₂ · g muscle¹ · min⁻¹)</td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>63.5 ± 4.8 (n = 21)</td>
<td>130.2 ± 7.4 * (n = 18)</td>
</tr>
<tr>
<td>0.5 Hz</td>
<td>172.0 ± 16.5 (n = 7)</td>
<td>255.7 ± 25.9 * (n = 6)</td>
</tr>
<tr>
<td>1.0 Hz</td>
<td>226.3 ± 18.5 (n = 7)</td>
<td>309.3 ± 18.9 * (n = 6)</td>
</tr>
<tr>
<td>2.0 Hz</td>
<td>256.3 ± 18.9 (n = 7)</td>
<td>364.9 ± 25.8 * (n = 6)</td>
</tr>
</tbody>
</table>

### Table 4
Respiratory flux as a function of stimulation frequency for wild-type and CK-deficient (MiM-CK⁻) SOL muscle. Values are means ± SE. *, P<0.05 compared to WT.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type SOL</th>
<th>MiM-CK⁻ SOL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol O₂ · g muscle¹ · min⁻¹)</td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>65.8 ± 9.3 (n = 21)</td>
<td>133.6 ± 11.0 * (n = 18)</td>
</tr>
<tr>
<td>1.5 Hz</td>
<td>248.6 ± 8.4 (n = 6)</td>
<td>265.6 ± 16.1 (n = 6)</td>
</tr>
<tr>
<td>3.0 Hz</td>
<td>285.4 ± 14.7 (n = 6)</td>
<td>310.7 ± 25.6 (n = 6)</td>
</tr>
</tbody>
</table>

Dynamic states

Figure 2 shows a typical trace of muscle respiration for WT (upper panel) and MiM-CK⁻ (lower panel) EDL muscle during a rest-stimulation-recovery experiment. At t=10 min, respiration was activated by 5 minute serial stimulation at 2 Hz. After stimulation was terminated respiration returned to basal rate in both muscles. Visual inspection of Figure 2 shows three major features: (i) the absence of any dramatic difference in response time of mitochondrial respiration to a step *increase* in contractile ATPase activity (τon) between WT and MiM-CK⁻ muscle; (ii) the presence of a dramatic difference in response time of mitochondrial respiration to a step *decrease* in contractile ATPase activity (τoff) between WT and MiM-CK⁻ muscle; (iii) the presence of a major difference between τon and τoff for WT muscle and, to a lesser extent, MiM-CK⁻ muscle. This typical observation was objectified by non-linear curve-fitting of exponential functions to the kinetic data (as exemplified by solid lines in Figure 2). Both mono- and double-exponential functions were used; however, statistically, no improvement of fit was obtained by using higher order than 1 (not shown).
Figure 3 shows the mean values (± SE) of \( \tau_{on} \) and \( \tau_{off} \) for WT and MiM-CK\(^{-/-}\) EDL and SOL muscles. For EDL, \( \tau_{on} \) and \( \tau_{off} \) were determined from serial twitch contractions at 1 and 2 Hz; no dependence on stimulation frequency was found neither for \( \tau_{on} \) nor \( \tau_{off} \) over this range of frequencies (not shown). For SOL, \( \tau_{on} \) and \( \tau_{off} \) were determined from serial twitch contractions at 3 Hz.

<table>
<thead>
<tr>
<th></th>
<th>Activation</th>
<th>Deactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EDL</td>
<td>69.4 ± 3.6 (n = 8)</td>
<td>264.1 ± 4.0 (n = 8)</td>
</tr>
<tr>
<td>MiM-CK(^{-/-}) EDL</td>
<td>54.0 ± 2.9 * (n = 5)</td>
<td>74.4 ± 4.0 * (n = 6)</td>
</tr>
<tr>
<td>Wild-type SOL</td>
<td>54.8 ± 1.5 # (n = 6)</td>
<td>162.6 ± 7.2 # (n = 5)</td>
</tr>
<tr>
<td>MiM-CK(^{-/-}) SOL</td>
<td>60.2 ± 3.3 (n = 6)</td>
<td>94.8 ± 8.8 * (n = 6)</td>
</tr>
</tbody>
</table>

*Table 5* Timeconstants of respiratory flux response during 0 Hz to 2 Hz and 0 Hz to 3 Hz activation and deactivation transitions for wild-type and CK-deficient EDL and SOL muscles. Values are means ± SE. *, \( P<0.05 \) compared to WT. #, \( P<0.05 \) compared to WT EDL.

We found that \( \tau_{on} \) was 69.4 ± 3.6 sec (n = 8) for WT EDL and 54.0 ± 2.9 sec (n = 5) for
MiM-CK−/−, respectively; these values were significantly different (P<0.05). Likewise, τ_{off} was significantly different for WT and MiM-CK−/− EDL (264.1 ± 4.0 sec (n = 8) versus 74.4 ± 4.0 sec (n = 6), respectively (P<0.01)). Importantly, within each muscle genotype τ_{on} and τ_{off} were significantly different (P<0.05); this difference was more pronounced in WT (Fig. 3). We found that τ_{on} was not significantly different in WT and MiM-CK−/− SOL muscle (54.8 ± 1.5 sec (n = 6) versus 60.2 ± 3.3 sec (n = 6)). In contrast, and analogous to our findings in EDL, τ_{off} was significantly different in WT and MiM-CK−/− SOL muscle (162.6 ± 7.2 sec (n = 5) versus 94.8 ± 8.8 sec (n = 6)). Analogous to the findings in EDL, within each muscle genotype τ_{on} and τ_{off} were significantly different (P<0.05), the difference being more pronounced in WT (Fig. 3). Comparison of τ_{on} and τ_{off} for EDL and SOL within each genotype revealed that for MiM-CK−/−, τ_{on} in EDL and SOL were not significantly different, as was the case for τ_{off} (Fig. 3). But in contrast, both τ_{on} and τ_{off} were significantly faster in SOL than in EDL for WT (P<0.05; Fig. 3).

**DISCUSSION**

The main novel finding of our study was that loss of CK function had no dramatic effect on the timeconstant of activation of mitochondrial respiration, both in EDL and SOL.
muscles. This was contrary to predictions. This result, together with other findings, is discussed below in the context of the ATPase network in muscle and its components.

**Stationary states of muscle respiration in WT and MiM-CK⁻⁻ EDL and SOL muscle**

**Basal respiration rate**

The increase in endogenous respiratory rate is in-line with the impaired free energy homeostasis [20, 21] and the increase in mitochondrial capacity reported for M-CK⁻⁻ fast-twitch muscle and MiM-CK⁻⁻ skeletal muscle [22, 23].

Quantitatively, this can be illustrated by calculating mitochondrial flux based on *in vitro* data of mitochondrial V_{max}, affinity of oxidative phosphorylation for ADP (K_{50}^{ADP}) and the free energy potential in WT and CK-deficient fast-twitch muscle. Measurements on isolated fast-twitch muscle mitochondria have shown a twofold increase in respiratory V_{max} and a 1.6-fold increase in K_{50}^{ADP} of oxidative phosphorylation, compared to mitochondria from WT gastrocnemius (Chapter 2). First we scaled the K_{50}^{ADP} values (3.5 ± 0.4 and 5.7 ± 0.7 µM for WT and MiM-CK⁻⁻, respectively) to WT skeletal muscle *in vivo* values (23-44 µM [24-26]) and converted the *in vitro* mitochondrial V_{max} rates to muscle V_{max} rates assuming 10.3 mg mitochondrial protein per gram of skeletal muscle tissue mass [27]. Next, we calculated the [ADP] at rest was estimated to be 15 and 49 µM in WT and MiM-CK⁻⁻ EDL, respectively.

Assuming second-order kinetics [26], the calculated respiratory flux for WT and CK-deficient EDL muscle was calculated to be 121.7 and 395.5 nmol O₂ · g muscle⁻¹ · min⁻¹, respectively. Although based on absolute numbers these results do not correlate with the respiratory values obtained in intact muscle, they do show the basis of the observed twofold increase in basal respiration in CK-deficient EDL, compared to WT (Table 3). These findings illustrate the effect of altered kinetics of mitochondrial function in combination with the muscle energy homeostasis at rest, possibly due to impaired Ca²⁺ metabolism [10].

The increase in mitochondrial ATP generating capacity can, therefore, be seen as an adaptive response to maintain high ATP/ADP ratios in EDL and SOL skeletal muscle.

**Activated respiration rates**

It has been reported that the respiratory V_{max} in M-CK⁻⁻ SOL muscle fibers is similar to WT, when measured at saturating ADP [22]. We have observed that MiM-CK⁻⁻ SOL and WT muscles have similar absolute respiration rates, upon stimulation at 1.5 and 3.0 Hz. These results show that contrary to MiM-CK⁻⁻ EDL muscles the respiratory V_{max} has not been increased in SOL muscles. However, due to decreased ATP/ADP ratios in MiM-CK⁻⁻ SOL muscle mitochondrial respiration operates at a higher basal level. Likewise, in MiM-CK⁻⁻ EDL muscle we observed increased basal respiratory rates at rest, compared to WT, but we also observed higher respiratory rates upon stimulation. This can be further illustrated by expressing the workload increase as a so-called duty cycle increase (relative to 0.5 Hz), as shown in the insert in Figure 1A. At increasing duty cycle the absolute increase in respiratory rate was similar for MiM-CK⁻⁻ and WT EDL muscle in the low duty cycle range. However, WT EDL muscle was less able to further increase its respiratory rate when stimulation was stepped-up from 1.0 to 2.0 Hz, likely due to the lower mitochondrial content, compared to MiM-CK⁻⁻. Since both
gastrocnemius and EDL muscle are fast-twitch muscles it is likely that the increase in mitochondrial content observed in MiM-CK⁻/⁻ hindleg muscle [16] and M-CK⁻/⁻ gastrocnemius fibers [22] also applies to EDL muscle. In addition to its increased basal rate of respiration, it is due to this enhanced mitochondrial capacity that oxidative phosphorylation in MiM-CK⁻/⁻ EDL muscle is better able to match myosin ATPase load, compared to WT EDL. We have recently obtained evidence for a similar contractile economy in WT and CK-deficient muscle, showing no alterations in the myosin ATPase load within the ATPase network of the genetically modified muscle cell (Chapter 6).

Furthermore, these findings confirm our prediction of similar operational domains for mitochondrial respiration in WT and CK-deficient fast-twitch muscle, derived from the observed adaptational shift to increased $V_{\text{max}}$ and $K_{50}^{\text{ADP}}$ of respiration in isolated mitochondria from MiM-CK⁻/⁻ gastrocnemius muscle (Chapter 2).

**Dynamic states of muscle respiration in WT and MiM-CK⁻/⁻ EDL and SOL muscle**

Mathematical modeling has provided a quantitative approach to study intracellular feedback signaling of high-energy phosphates between the sites of energy demand (myofibrils) and supply (oxidative phosphorylation). The classic temporal buffering role of CK was shown to dampen the response of mitochondrial activity to stepwise-changes in contractile activity to a great extend in these simulations, due to local buffering of ATP/ADP ratios by CK [12, 13]. This dampening function can be compared to the biocapacitor-like role of CK as described at submaximal stimulation rates by Meyer [13], in which PCr changes should follow mono-exponential kinetics characterized by a single apparent time constant for activation and de-activation of contractile ATPase.

Our results indicate that extrapolation of PCr kinetics to describe the mitochondrial response during transients in workload is not straightforward due to discrepancy that arises when one compares the kinetics of PCr hydrolysis and resynthesis, at the onset and end of contractions, respectively, to the kinetics of mitochondrial respiration during these transients. However, taking into account the asymmetry of CK dis-equilibration during the step-change in ATPase activity, faster respiratory kinetics were predicted at the onset of contractions, compared to end of contractions [12]. In WT EDL and SOL we indeed observed faster kinetics at the activation compared to de-activation of myosin ATPase load.

Furthermore, it was predicted that deletion of CK function would result in faster timeconstants of mitochondrial activity during step-changes in workload, due to the inability to temporarily dampen changes in ATP/ADP [12, 13].

**Timecourse of activation of respiration**

At the onset of contractions the rapid increase of myosin ATPase activity generates an error signal within the myocellular ATPase network. The first important factor in determining the speed at which the oxphos supply module can counterbalance the myosin ADP synthesis rate ($\tau_{\text{on}}$) is how fast this signal is transduced to the mitochondria. This signaling speed will depend on the diffusion distances between the sites of ATP demand and supply, with high mitochondrial packing giving shorter average diffusion distances [30]. In WT muscle, initiation of the ADP error signal will, furthermore, be dampened by the local biocapacitance of CK at the sites of ATP consumption [1].

Secondly, the functional properties of the supply module will determine its response to the error signal provided, i.e. the kinetic properties of mitochondrial oxidative phosphorylation ($V_{\text{max}}$ and the affinity of oxidative phosphorylation for ADP,
RESPIRATION OF WT AND CK-DEFICIENT MUSCLE

K_{50}^{ADP}) [12-14]. Slow-twitch SOL muscle has a twofold higher mitochondrial content compared to fast-twitch EDL muscle [22], thereby displaying faster kinetics of respiration at the onset of contractions. Furthermore, MiM-CK^- fast-twitch muscle has approximately twice the mitochondrial content compared to WT fast-twitch muscle, based on measurements of V_{max} of oxidative capacity in fast-twitch fibers [22]. Therefore, the prediction based on recent phosphorous NMR studies in M-CK deficient mouse gastrocnemius appears valid in that the kinetics of mitochondrial activity would be faster at the onset of contractions due to the upregulation of mitochondrial content in CK-deficient fast-twitch muscles, which would partly take over the buffering function of CK [14]. In our experiments, the increased mitochondrial content in CK-deficient EDL and WT SOL muscle does lead to a significantly shorter response time, compared to WT EDL.

Surprisingly, compared to the deactivation timeconstant, the response time of mitochondrial activation in WT and CK-deficient muscles is similar and appears not to be prolonged. This result is in contradiction with current modeling predictions and questions the effect of CK as a capacitor on delay of mitochondrial activity at the onset of muscle load transients. This either means that the on timeconstant of mitochondrial activity is faster in WT muscle compared to CK-deficient muscle or that the kinetics of respiration are relatively slow in the absence of CK function. It is important at this point to appreciate that the CK reaction will effectively facilitate diffusion of high-energy phosphates within the muscle cell [11]. Although diffusion of ADP will be lower in the absence of CK function, compared to other high concentration metabolites (e.g. ATP, PCR), [ADP] will still be in the µM range and be effective in providing an error signal to the highly ADP-sensitive mitochondria [11]. However, the lower diffusion of ADP may be of crucial importance based on recent evidence for the existence of diffusion gradients across the VDAC porins in mitochondrial outer membrane [31]. This gradient would have no effect on high concentration metabolites (creatine, ATP and PCR) but cytosolic ADP levels would need to be high to drive oxidative phosphorylation if activation would solely depend on ADP. The compartmentalized CK isozymes would effectively overcome this diffusion barrier by facilitated diffusion, communicating ATP/ADP changes to the intermembrane space compartment via PCR/creatine changes. This would speed-up the feedback communication between the myosin ATPase demand module and mitochondrial supply module [1].

If we consider facilitated diffusion by CK to be of crucial importance in signaling of high-energy phosphates in the muscle cell, CK deletion would obviously lead to slower activation kinetics of respiration at the onset of contractions. However, CK-deficient muscle is known to have increased glycolytic [10, 32] and adenylate kinase (AK) [22] capacity. The buffering role of AK was, furthermore, suggested on the basis of the increased AK catalyzed flux in the absence of CK function [33, 34]. In addition increasing mitochondrial volume in CK-deficient EDL muscle would lead to enhanced capacity to match myosin ATPase demand and would shorten diffusion distances within the muscle cell [30]. The combined effect of these factors may explain our observation of similar respiratory kinetics in WT and MiM-CK^- EDL muscle at the onset of myosin ATPase load. The alternative explanation could be that similar to heart muscle, ADP does not play a major role in the regulation of mitochondrial activity, but that regulation mainly takes place via Ca^{2+} [35]. However, fast-twitch muscles have a timescale of Ca^{2+} signal transduction in the millisecond range [28]. If Ca^{2+} were to be the main regulator of mitochondrial activity, timeconstants of mitochondrial respiration at the onset of
contractions would be in the range of seconds, not tenths of seconds (as we observe).

Timecourse of de-activation of respiration
At the end of contractions the kinetics of deactivation ($\tau_{\text{off}}$) was observed to be much faster in CK-deficient EDL and SOL muscle compared to WT. This observation corresponds well with the classic capacitor interpretation of CK-function, in which PCr resynthesis occurs during the initial phase of the rest period. This so-called ‘oxygen-debt’ provides a longer period of [ADP] elevation to repay the use of PCr during peak demand [3]. Phosphorous NMR studies have established that, unlike in wild-type muscle, PCr is essentially inert in CK-deficient muscle during periods of increased workload [10]. The absence of prolonged activation of mitochondrial respiration after the myosin ATPases have switched-off illustrates the lack of oxygen debt in CK-deficient muscle.

The effect of oxygen debt is, furthermore, illustrated by the faster $\tau_{\text{off}}$ for WT SOL compared to WT EDL. Slow-twitch SOL muscles have less biocapacitance, in the form of cytosolic CK, compared to fast-twitch muscles and are tuned to a greater duty cycle [3]. The faster timeconstant of deactivation in WT SOL muscles, compared to WT EDL, thus shows that SOL muscles rely on their CK-buffer to a lesser extent, compared to fast-twitch muscles. Interestingly, the deactivation timeconstant in CK-deficient EDL and SOL muscle was similar. This is indicative of similar ATP/ADP ratio buffering in these muscles, possibly provided by adenylate kinase activity [36].

In conclusion, our finding of increased basal rate of respiration in CK-deficient muscle underlines the effect of impaired energy homeostasis on muscle cell metabolic rate at rest. The increase in mitochondrial content observed in CK-deficient fast-twitch muscle makes higher absolute respiratory rates possible and enables both EDL and SOL knockout muscles to match similar contractile demands as WT. The loss of CK function in EDL and SOL muscle increases mitochondrial kinetics in response to step-changes in workload, indicative of the lack of a temporal energy buffer. However, the observation of similar mitochondrial activation times in response to onset of contractions emphasizes the facilitated diffusion present in WT muscle cells due to high CK activity at the sites of energy supply and demand. Specifically, it is suggested that CK activity in the mitochondrial inter membrane space compartment is vital for fast mitochondrial response to workload transitions.

Acknowledgements
The authors wish to acknowledge Drs. B. Wieringa, F. Oerlemans and K. Steeghs (Nijmegen University) for supplying the transgenic mice. We thank Dr. R.K. Porter (Trinity College Dublin) who put an OROBOROS oxygraph to our disposal. This research has been financially supported by The Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO).

REFERENCES


Chapter 6

INCREASED RESISTANCE TO FATIGUE IN CREATINE KINASE DEFICIENT MUSCLE IS NOT DUE TO IMPROVED CONTRACTILE ECONOMY

Frank ter Veld, Klaas Nicolay and Jeroen A.L. Jeneson

Submitted for publication
SUMMARY

There has been speculation on the source of increased endurance in CK-deficient muscle. An important factor may be the increased mitochondrial capacity and the reported alterations in MHC isoform composition in CK-deficient muscle. Furthermore, the absence of inorganic phosphate release from PCr hydrolysis in CK-deficient muscle has been postulated to lower fatigability in skeletal muscle.

In this study we tested the hypothesis that the reported shift in MHC composition to slower isoforms in CK-deficient muscle leads to a decrease in oxygen cost of twitch performance. In addition, we measured the timecourse of mechanical force recovery following a fatiguing series of twitches to test for possible alterations in excitation failure between wild-type and CK-deficient muscles.

To that aim, extensor digitorum longus (EDL) and soleus (SOL) muscles were isolated from wild-type (WT) and knock-out mice deficient in the cytoplasmic muscle-type and sarcomeric mitochondrial isoenzymes of CK, and we measured the amount of oxygen consumed per twitch time-tension-integral. In addition, we determined kinetics of twitch-force recovery after fatigue.

It is shown that the loss of CK-function does not alter the contractile economy of EDL and SOL muscles. Importantly, the kinetics of twitch-force recovery after 5 minutes of fatiguing stimulation were four- and twofold faster in CK-deficient EDL and SOL muscle, respectively, compared to WT.

It is concluded that the reported increase in endurance performance in CK-deficient muscles is mainly due to faster twitch-force recovery. We hypothesize that the reported increase in glycolytic capacity in CK-deficient muscle may be the source of the improved twitch-force recovery.
INTRODUCTION

During transients of muscle activity the creatine kinase (CK) reaction: phosphocreatine + ADP + H+ ↔ creatine + ATP is thought to minimize fluctuations in ATP/ADP ratio by acting as a spatial and temporal energy buffer [1]. By genetically deleting CK-function the impairment of local ATP/ADP buffering was shown to abolish the ability to perform burst-performance, impair free energy homeostasis and calcium handling [2]. However, low intensity stimulation revealed increased endurance performance compared to wild-type, despite of the fact that the buffering function of CK was absent [3-5].

There has been speculation on the origin of this increased stamina in CK-deficient muscle. An important factor may be the increased mitochondrial capacity on the energy supply side of muscle energetics [5, 6]. Another source may be the reported alterations in MHC isoform composition in CK-deficient muscle [2]. Inactivation of CK function using feeding with the creatine analogue β-guanidinopropionic acid (β-GPA) also showed a transition of MHC composition to slower isoforms, which was accompanied by a twofold improvement in oxidative contractile economy (defined as the amount of oxygen consumed per unit integral of tension and time) for mouse soleus (SOL) and extensor digitorum longus (EDL) muscle [7, 8]. Remarkably, Cr analogue feeding was observed to have no effect on the contractile economy of rat gastrocnemius, a predominantly fast-twitch muscle [9].

Recently, the effect of myoplasmic inorganic phosphate (Pi) on Ca2+ release has been proposed to play a major role in causing muscle fatigue, by lowering free [Ca2+] levels and thereby causing impaired excitation (for review see [10]). The absence of Pi release from PCr hydrolysis in CK-deficient muscle could thus be the main reason for the observed reduction in fatigability [3, 4].

In this study we examined in fast EDL and slow SOL muscle if the reported shift in MHC composition to slower isoforms observed in CK-deficient muscle [2, 11] leads to a decrease in oxygen cost of twitch performance. In addition, we tested for differential extents of excitation failure between wild-type and CK-deficient muscles (indirectly) by measuring the timecourse of mechanical force recovery following a fatiguing series of twitches.

EXPERIMENTAL

Animals and muscle preparation

Adult wild-type C57BL/6 mice were used as controls. Double knock-out mice, deficient in cytosolic muscle-type and sarcomeric mitochondrial CK (MiM-CK−/−), were generated in the laboratory of Dr B. Wieringa (Nijmegen University, The Netherlands) by gene-targeting as described previously [12, 13]. Offspring obtained in the breeding program was genotyped by PCR analysis on a regular basis. All experimental procedures were approved by the Committee on Animal Experiments of the University Medical Center Utrecht and complied with the principles of good laboratory animal care. Mice (age 21-30 days) were killed by cervical dislocation, EDL and SOL muscles of both hindlegs were prepared free from the surrounding tissue and a 5.0 silk suture (Ethicon, Norderstedt, Germany) was attached to the muscle tendons.
Muscle contractile performance

One end of the muscle was mounted in a custom-made oxygraph stopper and the other end was connected to an adjustable Harvard Apparatus 60-2995 force transducer (Harvard Instruments Limited, Edenbridge, UK), which allowed the muscle to be stretched to yield its maximum twitch force.

Figure 1 Typical result of twitch time tension integral (scaled to steady-state twitch-force prior to fatigue) during 5 minutes of 2 Hz (A) or 0.5 Hz (B) stimulation in wild-type (thick lines) and MiM-CK^−/− (thin lines) EDL muscle. Dashed lines represent 10% plus or minus deviation from average steady-state force, prior to fatigue. Insert in Figure A shows the small amount of ‘burst’ performance of MiM-CK^−/− EDL initiated after approx. 10 seconds into the stimulation protocol.
Muscles were stimulated via platinum wire electrodes by supramaximal pulses (0.5 ms duration; 6-15 V) by a Grass S88 dual channel stimulator (Astro-med, West Warwick, RI, USA) and force signals were digitized at 1000 Hz sampling rate. The stimulation protocol started with 10 minutes of rest to record basal respiratory flux followed by 5 minutes at 0.5, 1.0 or 2.0 Hz and 1.5 or 3.0 Hz for EDL and SOL muscles, respectively. Episodes of muscle stimulation were separated by 10 minute intervals during which non-stimulated basal respiration of the muscle was recorded. Following the 5 minutes of stimulation, the timeconstant of twitch-force recovery was recorded using a low frequency stimulation protocol consisting of 1 minute at 0.2 Hz (0.4 Hz for MiM-CK⁻/⁻ muscles) followed by 9 minutes at 0.02 Hz. Basal muscle respiration was recorded simultaneously.

**Oxygen consumption of intact EDL and SOL muscle**

The rates of oxygen consumption (nmol O₂·g muscle weight⁻¹·min⁻¹) were determined at 20 °C, using a high-resolution oxygraph (OROBOROS, Innsbruck, Austria) with a stirring rate of 500 rpm. Respiratory fluxes were corrected for chamber oxygen leak based on an exponential decay of P_{O2} in the oxygraph chamber at 20 °C containing Ringer solution (116 mM NaCl, 25.3 mM NaHCO₃, 4.6 mM KCl, 2.5 mM CaCl₂, 1.16 mM KH₂PO₄, 1.16 mM MgSO₄, pH 7.4) according to [14]. Oxygen solubility of 5 % CO₂ – 95 % O₂ equilibrated Ringer medium was calculated according to [15] and oxygen electrode response time was constant at 4 seconds (tested prior to each
experiment). All measurements of muscle respiration were performed after 30 minutes of equilibration, to avoid measurements at high chamber oxygen-leak. All measurements of muscle respiration were performed as randomized paired-experiments with simultaneous measurement of one WT and one MiM-CK-/- muscle in a dual-chamber setup. To avoid oxygen limitation of respiration in SOL and EDL muscles at 20 °C [16] all measurements were performed above a P O2 of 450 Torr. Chamber volume (approx. 5.2 mL) and muscle weight (blotted and tendon free) were determined after each experiment.

**Data acquisition, analysis and statistics**

Oxygraph and force transducer data-acquisition was performed with LabView software (National Instruments, Woerden, The Netherlands). Muscle mechanics data was analyzed using LabView software (with analysis programs generously provided by R.K. Gronka, Department of Radiology, University of Washington, Seattle, U.S.A). Timeconstants of force recovery, time-tension-integrals of twitch force and concomitant oxygen consumption during the 5 minute stimulation periods were calculated using Origin 6.0 (Microcal Software Inc., Northhampton, MA, U.S.A.). Reported data are presented as arithmetic means ± SE. Statistical analyses were performed using a Student’s unpaired t-test. Differences between MiM-CK-/- and WT muscle were considered significant if P<0.05.

**RESULTS**

**Mechanical performance**

Initial twitch force per mg of muscle wet-weight was slightly lower in MiM-CK-/- EDL muscles and significantly lower in MiM-CK-/- SOL muscles, compared to WT (data not shown). The half rise time to peak for MiM-CK-/- EDL and SOL muscles was found to be similar to WT (data not shown). However, the half time of relaxation was significantly longer in CK-deficient EDL (51.1 ± 2.4 ms (n = 4)), compared to WT (35.8 ± 5.8 ms (n = 6)). CK-deficient SOL muscle also displayed prolonged half relaxation times. This parameter amounted to 166.8 ± 8.9 (n = 8) and 225.6 ± 19.3 (n = 7) ms for WT and CK-deficient SOL, respectively.

Figure 1A displays a typical EDL stimulation experiment. Tension-time-integral (TTI) values were scaled to average steady-state twitch-force prior to fatigue. During the first phase of 2 Hz stimulation, WT EDL (thick line) showed typical burst performance, while CK-deficient EDL (thin line) lacked burst performance. Interestingly, a sort of force output burst was observed in CK-deficient EDL (Fig. 1A, insert). However, after steady-state twitch performance, lasting 20-40 sec, MiM-CK-/- EDL displayed increased endurance performance, compared to WT. At 0.5 Hz stimulation no difference in fatigue was observed between MiM-CK-/- and WT EDL muscles (Fig. 1B). Burst activity was, however, absent in CK-deficient EDL. After stimulation was stopped a low frequency of stimulation, 1 minute at 0.2 and 0.4 Hz, for WT and MiM-CK-/-, respectively, followed by 9 minutes at 0.02 Hz, was applied to estimate the speed of twitch-force recovery. Figure 2 shows that a much faster twitch-force recovery occurred in MiM-CK-/- EDL. Similar to CK-deficient EDL muscles, although less pronounced, MiM-CK-/- SOL displayed enhanced endurance (data not shown).
Figure 3 Total amount of oxygen consumed versus twitch time-tension-integral during 5 minutes of stimulation in wild-type (closed symbols) and CK-deficient (open symbols) EDL (squares) and SOL (circles) muscle. Best linear fits to the data points are indicated as solid and dashed lines for wild-type and CK-deficient muscle, respectively. Insert shows consumed oxygen during the recovery period. The schematic illustrates the activation (start) of respiratory flux and the difference in total oxygen consumed during the recovery phase and total amount of oxygen consumed. The dashed line in the schematic shows the more rapid decline in respiratory flux in CK-deficient EDL after terminating muscle stimulation (end).
Contractile economy of CK-deficient muscle

The contractile economy of isometric twitch-force was estimated by measuring the total amount of oxygen consumed during 5 minutes of stimulation. As an example, Figure 2 shows the twitch-force performance along with the oxygen consumption rate in WT and MiM-CK⁻/⁻ EDL muscles. The start of the 2 Hz stimulation protocol at t=300 sec led to activation of mitochondrial oxidative phosphorylation. The 300 seconds of 2 Hz stimulation induced fatigue in WT and MiM-CK⁻/⁻ EDL muscle. CK-deficient EDL twitch-force reached a plateau while twitch-force decline was ongoing in WT EDL. At the end of contractions, MiM-CK⁻/⁻ EDL showed much faster twitch-force recovery. Additionally, the timecourse of respiration deactivation was faster during the recovery phase.

Contractile economy can be estimated by plotting the total amount of oxygen consumed during muscle activity against the time-tension-integral (TTI) of all individual twitches [7] during the 5 minute stimulation period (Fig. 3). The isometric economy was found to be identical in WT and MiM-CK⁻/⁻ EDL, based on the similar slope of 26.2 ± 1.4 and 23.0 ± 1.2 µmol O₂ · Ns⁻¹ for WT and MiM-CK⁻/⁻ EDL, respectively (Fig. 3). The total amount of oxygen consumed per TTI in WT SOL muscles was 6.04 ± 0.37 µmol O₂ · Ns⁻¹ which was not significantly different from MiM-CK⁻/⁻ SOL muscles (4.98 ± 0.39 µmol O₂ · Ns⁻¹). The insert in Figure 3 displays total oxygen consumption during the recovery phase, after muscle stimulation was stopped (illustrated by the schematic in Figure 3, marking the oxygen consumption during the recovery phase). The timecourse of oxygen consumption during this recovery phase shows the effect of faster
mitochondrial response times on the total amount of oxygen consumed during recovery (illustrated by the dashed line in schematic of Figure 3, indicating the faster timecourse of CK-deficient muscle oxygen consumption returning to basal respiratory rate).

**Timeconstants of poststimulation twitch-force recovery**
To explore the rate of recovery following stimulation at 2 Hz and 3 Hz to induce fatigue in EDL and SOL, respectively, we sampled peak twitch-force at a low frequency, 0.2 and 0.4 Hz during the first minute of force recovery for WT and MiM-CK− muscles, respectively. The final phase of recovery was sampled at 0.02 Hz. It was observed that post-stimulation twitch-force recovery was fast in CK-deficient muscle relative to WT muscles (Fig. 2). Two Hz stimulation induced a similar degree of fatigue in WT and MiM-CK− EDL, i.e. peak twitch-force decline was 39.4 ± 3.1 (n = 7) and 43.2 ± 4.6 (n = 5) % of initial force, respectively. CK-deficient EDL force recovery was fourfold faster than in WT (with recovery timeconstants of 28 ± 5 (n = 9) and 108 ± 11 (n = 7) seconds for MiM-CK− and WT EDL muscles, respectively) (Fig. 4). WT SOL muscles displayed significantly greater force decline after 5 minutes at 3 Hz compared to CK-deficient SOL muscles (48.2 ± 3.4 (n = 8) and 60.8 ± 3.0 % (n = 7) of initial for WT and MiM-CK− SOL, respectively). Similar to EDL muscle, the timeconstant of force recovery was slower in wild-type than to CK-deficient SOL muscle (115 ± 14 (n = 10) compared to 56 ± 9 seconds (n = 4) for WT and MiM-CK− SOL, respectively) (Fig. 4).

**DISCUSSION**
The two main findings of this study are the identical oxidative energy demand by the myosin ATPase machinery, based on similar isometric twitch force economy, in CK-deficient muscle and the rapid twitch-force recovery in CK-deficient EDL and SOL muscles, compared to wild-type.

**Contractile economy in MiM-CK− EDL and SOL muscle**
Many physiological studies that addressed the loss of creatine kinase function in muscle have measured the myosin heavy chain (MHC) composition to explore possible adaptive alterations in the energy demand of the muscle contractile apparatus. In muscle lacking a fully functional CK system, either due to β–guanidinopropionic acid (β-GPA) feeding [8, 9, 17, 18] or by deletion of CK enzymes [2, 19] a shift to slower MHC isoforms (type I and IIa increase) has been observed in fast-twitch gastrocnemius and EDL muscles.

The energetic cost to maintain tension is known to depend on the myosin isoform composition due to intrinsic differences in the cross-bridge cycle rates and the myosin ATPase activities [16, 20, 21]. However, depending on the type and duration of muscle work performed (e.g. twitch or tetanus) the contribution of other components, such as Ca2+ handling, in determining the total energetic cost may vary [22]. Moerland et al. studied the contractile economy based on the poststimulatory oxygen consumption during recovery from EDL and SOL muscles of β-GPA fed mice and observed a 50% decrease in energetic cost to maintain tetanus tension in both muscle-types [7]. They related their findings to the more cost-efficient myosins present in these β-GPA treated muscles [8] and concluded that the observed myosin shift led to a twofold increase in contractile economy.
Based on these findings and the similar shift in MHC composition observed in CK-deficient and β-GPA treated fast-twitch muscles we expected to find a similar increased contractile economy in MiM-CK-/- EDL and SOL muscle. In contrast however, we observed a similar energetic cost in CK-deficient and WT EDL as well as SOL muscle based on total oxygen consumption during 5 minutes of submaximal twitch-stimulation (Fig. 3). At first glance this appears in contradiction with the findings of Moerland et al. [7]. However, at closer inspection the measurement of oxygen consumption during recovery reveals a similar contractile economy increase as observed in β-GPA treated muscles (Fig. 3, insert).

Although β-GPA treated and CK-deficient fast-twitch muscles display similar shifts in MHC IIa/IIb composition, the end-effect on contractile economy may be less dramatic when taking into account that the reduction in ATP cost of contractions is only two- to threefold when one compares IIa to IIb fibers [20, 21]. Therefore, to estimate the end-effect of alterations in MHC isoform composition, we calculated contractile economy for isometric twitch-force based on tension-cost values for rat single fibers provided by Bottinelli et al. [20]. An increase in IIa/IIb ratio of 100% observed for CK-deficient fast-twitch muscle, would improve contractile economy by only 10% (Fig. 5). This calculation includes the minor increase in type I MHC. Based on the reported shifts.

Figure 5 Calculated increase in MHC IIa/IIa ratio in fast-twitch-muscle due to loss of CK-function improves estimated contractile economy. The figure is based on reported shifts in MHC composition for fast-twitch muscles upon loss of CK function [2, 9, 11, 31] and tension-cost values for rat single fibers [20]. Solid line represents least-squares regression (% estimated contractile economy increase = 0.091 \cdot % MHC IIa/IIb ratio increase, compared to WT).
in MHC composition in CK-deficient muscles it was, therefore, not expected to find increased economy (even with extremely low noise on the experimental data-points). Furthermore, based on phosphorous NMR measurements on fast-twitch gastrocnemius muscle from β-GPA fed rats the ATP cost of either twitch or tetanus was found to be similar to wild-type muscle [9].

It appears that the reported shifts in MHC composition in CK-deficient muscles do not increase contractile economy, however, the increase in slower MHC isoforms does lead to longer twitch times (as shown by increased half relaxation times, see results section).

MiM-CK deficiency leads to a threefold faster return of mitochondrial oxidative flux to basal respiration (Chapter 5). This absence of prolonged activation of mitochondrial respiration following stimulation illustrates the lack of so-called ‘oxygen debt’ in CK-deficient muscle during which the muscle ‘repays’ the use of PCr during peak demand [23]. Phosphorous NMR studies have established that, unlike in wild-type muscle, PCr is inert in CK-deficient muscle during periods of increased workload [5]. Oxygen consumption thus lacks the ‘oxygen-debt’ component during recovery in CK-deficient muscle and is, therefore, faster, explaining our result. By only measuring oxygen consumption during recovery one, therefore, underestimates total oxygen cost in the absence of CK-function. Again, illustrated by the insert in Figure 3 showing reduced recovery oxygen consumption for CK-deficient muscle while the total oxygen cost plotted in Figure 3 was similar for CK-deficient and WT muscle.

**Twitch-force recovery in MiM-CK−/− EDL and SOL muscle**

As a first approach the use of measuring force as an indicator of [Ca2+] in muscle constitutes a well-established and common practice [24]. Visual inspection of Figure 1A confirms previous reports on fatigue behavior in CK-deficient muscle. At the onset of contractions twitch-force rapidly declined in MiM-CK−/− muscle compared to WT, indicative of impaired burst-performance. Endurance performance was, however, improved compared to WT as can be seen from the plateau in twitch-force that was reached in CK-deficient muscle, compared to the ongoing twitch-force decline in WT muscle (Fig. 2).

Our finding of extremely rapid recovery of twitch-force in EDL and SOL muscle after 5 minutes of fatiguing stimulation was unexpected based on the impaired free energy homeostasis [3, 4, 25] and Ca2+ handling [5] reported for CK-deficient muscle.

Mechanical studies on the effects of CK-deficiency on muscle fatigue have primarily relied on tetanus performance and have revealed decreased burst performance and improved endurance performance [3-5]. The enhanced rate at which CK-deficient muscle recovers from twitch-force fatigue has important implications for the interpretation of mechanics studies on MiM-CK−/− muscles. In experiments that use tetanus to induce muscle fatigue the use of a tetanic interval of 1-3 seconds is common practice. However, with force recovery being fourfold faster in CK-deficient EDL muscle, the apparent endurance performance is greatly dependent on the choice of tetanic interval length. This observation was recently made by Dahlstedt and coworkers [3]. The possible mechanism that was put forward by Dahlstedt et al. to explain increased stamina in CK-deficient muscle was based on the lack of myoplasmic inorganic phosphate build-up, due to the absence of PCr hydrolysis during muscle work [3, 4]. This same mechanism may also apply to our observation of faster twitch-force
recovery. P_i-induced reduction of crossbridge force and inhibition of Ca^{2+} release from the sarcoplasmatic reticulum will be prevented if PCr hydrolysis can no longer occur. It should, however, be pointed out that although no fatigue was observed in WT EDL at 0.5 Hz stimulation (Fig. 1B), PCr is hydrolyzed by CK [26]. In this case the formation of P_i apparently has no effect on muscle performance, questioning the proposed effect of P_i on Ca^{2+} release and fatigability.

However, other mechanisms should also be considered when attempting to explain faster force recovery. Particularly, the increase in glycolytic capacity in CK-deficient muscle [5, 19] may have a profound effect on twitch-force recovery by increasing energy supply to muscle cell cation-homeostasis (i.e. Ca^{2+}, K^+). Some evidence exists for smooth and cardiac muscle that a major part of poststimulation energy demand also depends on glycolytic ATP [27, 28]. However, no such evidence exists for skeletal muscle as yet. We should, furthermore, consider the effect of enhanced glycolytic capacity on Na^+/K^+-pump capacity. This enzyme also depends on glycolytic ATP [29] and the increased glycolytic capacity observed in CK-deficient muscle could lead to a more rapid restoration of the action potential after muscle fatigue. Furthermore, inspection of Figure 2 reveals that respiratory flux had not returned to basal levels when force had already recovered to initial values, strengthening our hypothesis of glycolytic fueling of the cation pumps. Another observation that supports the increased role of glycolytic ATP supply in CK-deficient muscle during stimulation is the reported decrease in burst-performance [2]. The slow activation of glycolysis would hamper burst activity.

The implications of our findings for the understanding of the plasticity of the muscle ATPase network are that cellular adaptations to counterbalance impaired energy homeostasis, due to deletion of CK-function, mainly take place at the supply level (e.g. mitochondrial capacity is enhanced [5, 6, 30]). We have shown that the deletion of CK-function does not alter the contractile economy of EDL and SOL muscles, although the MHC composition has shifted to slower isoforms. This is indicative of minor alterations on the ATP demand side within the skeletal muscle ATPase network. Furthermore, we conclude that the reported increase in endurance performance in CK-deficient muscles is mainly due to a faster twitch-force recovery and that glycolysis may be a more dominant supplier of ATP.

Acknowledgements
The authors wish to acknowledge Drs. B. Wieringa, F. Oerlemans and K. Steeghs (Nijmegen University) for supplying the transgenic mice. We thank Dr. R.K. Porter (Trinity College Dublin) who put an OROBOROS oxygraph to our disposal. We are grateful to A. Doornenbal (Utrecht University) for expert help in force mechanics analysis. This research has been financially supported by The Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO).

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Chapter 6


Chapter 7

Summarizing Discussion
Muscle plasticity

This study has provided insight into the adaptive strategies of the muscle cell in response to creatine kinase deficiency. Figure 1 shows a schematic of muscle functional (force) output. The basis of sustained mechanical output is the balance between ATP free energy supply (oxidative phosphorylation, glycolysis, and CK/AK buffers) and demand (the ATPases of the contractile elements and cation pumps) within the muscle cell. In the case of ablation of CK function, the muscle cell is apparently able to adjust its supply components in order to maintain energy balance. This conclusion is based on the findings of increased mitochondrial capacity (Chapter 2) and increased mitochondrial respiration in intact muscle (Chapter 5). The ATP free energy demand component, however, in the muscle cell ATPase network is not altered in response to CK-deficiency, as evidenced by the similar contractile economy in wild-type and CK-deficient muscle (Chapter 6).

The issue of chronic lowering of the cell’s phosphorylation potential and the subsequent alterations in contractile as well as metabolic proteins was previously raised by Sweeney [1]. He argued that the genetic programs would not allow for dissociation of contractile and metabolic protein expression in muscle. However, recent work by Wu et al. has elegantly shown a calcium-regulated signaling pathway that specifically controls mitochondrial biogenesis in mammalian cells [2]. This demonstrated that upregulation of mitochondrial capacity can indeed occur independently of contractile protein expression.

Important data on alterations in the biological hardware in response to CK deletion has come from the lab of Wieringa by characterizing the muscle cell at the proteomics level [3]. This study showed that minor alterations occur in the expression of contractile proteins, while glycolytic and mitochondrial proteins exhibit a marked increase in CK-deficient skeletal muscle. This thesis provides physiological evidence for increased mitochondrial energy supply and unchanged contractile energy demand in in vivo skeletal muscle.

Operational domain of mitochondrial flux

Many studies on adaptations due to CK deletion were restricted to measuring the maximal capacity (V\text{max}) of mitochondrial respiration, while aspects of regulation received little attention. However, the importance of both regulability as well as slope in determining the actual flux has been elegantly emphasized by Van Dam and Westerhoff [4]. The scope is set by the available capacity of the enzyme/pathway (V\text{max}) and the regulation of this flux is determined by the affinity constant(s) (K_{50}). The analysis in Chapter 2 shows that adaptive changes in both parameters occur to support energy balance and function in response to CK-deficiency. We observed an increase in respiratory capacity and a concomitant decrease in affinity of oxidative phosphorylation for ADP for (Mi)M-CK deficient fast-twitch muscle. These kinetic adaptations were shown to rescue both absolute capacity to generate ATP via oxidative phosphorylation as well as offer linear robust control of the cytosolic ATP free energy potential during workload increments.

Role of (Mi-)CK in intracellular high-energy phosphate signaling

The precise mechanism by which CK communicates contractile activity to the mitochondria is not fully understood.
The two main theories are based on either the ‘phosphocreatine-shuttle’, in which the intracellular communication solely relies on PCr and Cr flux [5-7], or on a so-called CK-mediated facilitated diffusion flux by Cr and ADP [8]. There is, however, agreement on the classical textbook role of CK as a temporal energy buffer. An important argument against the need for a shuttle system has been the absence of reported differences in diffusion for ADP and Cr between the sites of energy demand and supply. As described by Meyer in 1984: “It does not seem reasonable to refer to the specific ‘channeling’ or ‘shuttling’ of high-energy phosphate by PCr, terms which implicitly connotate a novel mechanism, unless it can be shown that adenylate diffusion is restricted in comparison to creatine, and this has not been demonstrated.” This statement was true at that time, because although VDAC had already been described [9], its function and kinetics were only poorly understood. Evidence that ADP permeation across the mitochondrial outer membrane could be controlled by VDAC was provided in 1996 [10] and it was not until 2002 that Gellerich et al. reported flux dependent diffusion gradients up to 18 µM for ADP to exist over the mitochondrial outer membrane [11]. Mathematical model computations, including our own [12] (Chapter 3), support the role of VDAC in limiting the ADP flux from the cytosol to the mitochondrial inter membrane space. Furthermore, based on two-compartment modeling, with an assumed diffusion restriction of 30 µM between the cytosol and mitochondrial compartment, over 80% of the ADP error signal would be communicated to the mitochondria by changes in PCr/Cr ratio (Dr. O. Kongas, Tallinn Technical University, Tallinn, Estonia, personal communication).

Kushmerick and Meyer have modeled the mitochondrial supply component as a single compartment, with no dimensions and consequently predicted near-instant activation of mitochondria in CK-deficient muscle, as the change in ADP level is not buffered by CK [13, 14]. However, our measurements in intact muscle (Chapter 5) are at variance with the prediction of near-instantaneous activation of respiration in CK-deficient muscle upon a step-wise increase in workload. So although the ADP error signal from the contractile elements to the mitochondria is dampened by CK activity in WT muscle, the activation time of respiration is equally short as in muscle without (CK) dampening. In light of the ‘shuttle’ versus ‘facilitated diffusion’ function of CK this means that CK-deficient muscle can do well without vectorial transport of PCr/Cr if ADP/ATP signaling alone provides fast communication between the sites of energy demand and supply, provided mitochondrial capacity is doubled. This shows that exclusive shuttling of PCr and Cr between the myosin ATPase and mitochondrial ATP synthase is not a prerequisite for intracellular communication, which argues against the shuttle concept in its extreme form.

On the other hand our finding that activation times of mitochondrial flux are equal to those observed in CK-deficient muscle, lacking temporal dampening, illustrate
the advantage of having CK activity in two compartments. If diffusive flux of low concentration metabolites, e.g. ADP, from the cytosol to the mitochondrial inter membrane space compartment is indeed restricted by VDAC under in vivo situations, shuttling of PCr/Cr and also facilitated diffusion by CK would ‘overcome’ the mitochondrial outer membrane barrier due to the relatively high concentrations of these metabolites (10-40 mM), compared to ADP (in the micromolar range). A crucial experiment to perform in future studies is to measure mitochondrial activation times in Mi-CK deficient muscles. These muscles are unable to translate cytosolic PCr/Cr changes within the inter membrane space compartment to changes in ATP/ADP and are, therefore, expected to have slower activation kinetics, if signaling speeds are indeed increased by the tandem function of cytosolic and mitochondrial CK.

Sources of alternative ATP supply in CK-deficient muscle

Wieringa and coworkers have reported an increased glycolytic capacity in muscle in response to CK-deficiency [3]. The importance of glycolytic ATP supply was recently put forward by Shulman who proposed the so-called ‘glycogen-shunt’ where activation of glycolysis would be in the millisecond range [15]. In Chapter 5 we observed relatively slow activation of mitochondrial respiration in CK-deficient muscle upon onset of contractions, considering all ADP signal dampening by CK would be lost in these muscles. This could be explained by the reported increased flux through adenylate kinase [16] that could dampen the ADP error signal. Alternatively, signal dampening at the myofibrils could be achieved by rapid activation of glycolytic activity [15]. A prerequisite for ADP dampening by glycolysis would be rapid activation of this flux, which is currently not a widely accepted concept [17].

In this respect the observations of more rapid force recovery (Chapter 6) and enhanced fatigue resistance in CK-deficient muscles [18-21] are also of relevance. Although both alterations in contractile performance can partly be explained by increased mitochondrial capacity, the observed timecourse of recovery of force is not reflected in parallel changes in mitochondrial activity. This argues for enhanced glycolytic ATP supply in CK-deficient muscle. A third point is the observation of delayed ‘mini burst performance’ in CK-deficient EDL muscle described in Chapter 6. These muscles lack the full burst activity of WT muscles but a small increase in force output is observed. We hypothesize that this small burst, initiated at approximately 10 seconds into the stimulation protocol, represents the delayed activation of glycolytic flux, which would imply that activation of glycolysis is in the order of seconds [17] and not milliseconds [15]. To test this assumption, the formation of lactate should be measured in future experiments to determine glycolytic flux. Furthermore, the use of specific adenylate kinase inhibitors may clarify its buffering role during workload transients in creatine kinase deficient muscle.

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SAMENVATTING

De balans tussen energie vraag en aanbod, in de vorm van ATP, is essentieel in spiercellen. Het creatine kinase systeem vervult in spiercellen een zowel bufferende als transporterende rol, waardoor fluctuaties in ATP-energie behoeftte kunnen worden opgevangen. Het verwijderen van de creatine kinase eiwitten in muizenspiercellen met behulp van genetische manipulatie wordt onderzocht in dit proefschrift. Alhoewel het verwijderen van de creatine kinase enzymen niet dodelijk is in muizen treden er veranderingen op in de energie balans. Vooral bij intensieve spier inspanning wordt de verminderde mechanische functie in spieren zonder creatine kinase duidelijk.

In hoofdstuk 2 van dit proefschrift worden de veranderingen in mitochondriële functie bestudeerd in langzaam- (b.v. hart) en snel-samentrekkende (b.v. kuitspier) dwarsgestreepte spieren uit creatine kinase deficiënte muizen. De resultaten van deze studie laten zien dat de ATP-energie genererende capaciteit in snel-samentrekkende gastrocnemius spier verdubbeld indien creatine kinase afwezig is. Tevens wordt deze ATP-energie genererende activiteit minder gevoelig voor veranderingen in de energie balans. Deze verlaagde gevoeligheid zou er op kunnen duiden dat gastrocnemius spiercellen zonder creatine kinase zich hebben aangepast om regulatie te behouden bij een gereduceerde ATP-energie balans.

De regulatie van mitochondriële functie wordt in hoofdstuk 3 nader bestudeerd met behulp van eigen meetgegevens en mathematische modellering. De communicatie tussen de mitochondriën en het cytosol van de spiercel blijkt op basis van de modellering sterk verhoogd indien (cytosolisch) creatine kinase niet aanwezig is in de hartspier, misschien door meer kanaal vormende “porine” eiwitten in de buitenmembraan van de mitochondriën.

In hoofdstuk 4 wordt de diffusie van metabolieten in zogenaamde gepermeabiliseerde spiervezels bestudeerd. Er wordt geconcludeerd dat deze diffusie sterk is gereduceerd en dat de eigenschappen van de mitochondriën in deze vezels niet wezenlijk anders zijn dan van geïsoleerde mitochondriën.

In hoofdstuk 5 wordt deletie van creatine kinase bestudeerd in intacte spieren. Het ontbreken van deze enzymen leidt tot een versnelde activatie van mitochondriële ATP-energie productie tijdens spierstimulatie. Tevens is de ATP-energie genererende capaciteit verhoogd in snel-samentrekkende spier indien creatine kinase afwezig is. In zowel langzaam als snel samentrekkende spier is de mitochondriële activiteit verhoogd tijdens rust, wat de verslechterde energie balans in creatine kinase deficiënte spieren bevestigd.

De mechanische functie van muizenspier zonder creatine kinase wordt nader bestudeerd in hoofdstuk 6. De studie toont aan dat na kracht afname door spier vermoeidheid de spieren zonder creatine kinase sneller herstellen en kracht kunnen leveren in vergelijking met normale spieren.

Concluderend kan worden gesteld dat de energie vraag in spieren zonder creatine kinase niet anders is in vergelijking met normale spieren. De voornaamste verandering treedt op in de ATP-energie aanbod component, namelijk de mitochondriële capaciteit. Door een verhoogde energie genererende capaciteit lijkt een spiercel zonder creatine kinase zijn taak te kunnen blijven vervullen, een plotselinge toename in spierinspanning is echter niet mogelijk in deze spieren. Aanhoudende spier activiteit blijkt echter beter door de verhoogde mitochondriële capaciteit.

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DANKWOORD

Een promotie-traject kent vele fasen en dus zijn er vele dankwoorden denkbaar. Een versie:


Beste Klaas, de basis van dit proefschrift ligt bij jou. Maar zoals altijd gedragen mutante muizen zich niet conform een project-voorstel, wat tot menig frustratie heeft geleid. Ik denk dat ik jou vooral moet bedanken voor je vertrouwen, vanzelfsprekend was dit niet altijd. Bedankt voor al je steun Klaas en heel veel succes in Eindhoven.

Max, van jou kwamen de “final nudges” om het project af te ronden. Het project stond ver van je af maar jouw steun is cruciaal geweest.

 Dit boekje heeft verder uit vele hoeken steun mogen ontvangen. Cees van Echteld hartelijk dank voor het lab gebruik en gastvrijheid van de “cardio’s”. Beste naamgenoot Frank Bruggeman, nog zo’n “quick-mind” van de VU... dank voor de vele gesprekken en modellering hulp voor deze wiskunde-nul. Lodewijk Thielens, zonder veternaire biochemie geen mooie mito suspensies, dank voor het centrifuge gebruik! Frank Gellerich Herzlichen Dank für die Hilfe und Gastfreundschaft in Halle. Well Richie, you’ve been a boost through-out. In the end you even supplied the tools. Cheers to Dublin and I really hope to see you and family soon. Thanks Richie! Voor de uitmuntende dierverzorging moet ik vooral Anja, Els en Joyce bedanken. Het GDL maakt het jullie niet makkelijk maar jullie doen een top-job!

 Dit boekje zou er ook niet zijn zonder alle plezier en gekheid van de kollega’s... het was soms TE gezellig. Beste Marijn, onder dat brave exterieur van jou gaat veel schuil. Dank voor je steun en pep-talks. We zullen elkaar verder door de afdeling en dan kom je altijd Marcel tegen. Altijd. There’s nothing better than the real thing. Echt Marcel dus. Heb van je genoten Marcel. Zoals zijn er maar weinigen. Niet vergeten. In 1998 onthoort je dan de “three-amigos”... Rick (de vragen hebben nog nooit zoveel antwoorden bevat), Kees (jij loopt bij een ander dwars door het hoofd/hart) en Robin (briljant en nog veel meer). En (voormalig “Beertje”) Blezer, jouw gevoel voor humor is absoluut zalig en grenzenloos, ik zal je Wetten Daß opmerking nooit vergeten, een paar andere opmerkingen helaas ook niet. Have fun Jeanette en Erwin! Tuurlijk was er meer vrouwelijke versterking. Lieve Maaike, nog een vreemde vogel binnen in-vivo, dank voor je wijze woorden en vertrouwen. Dan het culturele licht van de taakgroep, jawel Janneke ‘t was lachen hoor! Je maakte het gezellig en voor je “drive” heb ik veel respect. Vaak zijn accenten/dialecten “dead-give-aways” maar om Wouter een Groninger stempel toe te kennen wordt lastig, ja. Of toch, ja? Maar als non-import hebben we Gerard, Utrechter in hart en nieren, je “beide-benen-op-de-grond” aanpak is vaak net wat een AiO nodig heeft. Oh ja: en gouden vingers! And how cold our Northern hearts can sometimes be, Pedro, thank you for being Spanish! I did feel humble at times... the warmth and hospitality of you and Monse is not our way. Together with our “cuisine” this is a major Dutch shortcoming.
Natuurlijk speelt zich niet alles op het lab af. Lieve Haukeline, je steun in Utrecht was super. Zal in Hoogkerk zeker niet veranderen. In de laatste fase was jij zo dichtbij lieve Ingeborg, duizend maal dank voor je steun gedurende deze tijd! The homefront in Groningen... veel heb je moeten aanhoren Linda, je bent een topper! Gouden hart heb je, meer van jou geleerd dan er in dit boekje staat. Het beste voor jou, Klaas en de kleine gewenst. Beste Atti, bedankt voor je luisterende oor! We zijn d’r voor elkaar. En Henk, je was d’r altijd voor me. Heb veel aan je gehad. Evert, trouwe toeverlaat.

Heel veel dank.
CURRICULUM VITAE

In 1998 begon hij als onderzoeker in opleiding bij de afdeling Experimental In-Vivo NMR van het Image Sciences Institute aan het University Medical Center Utrecht. Daar verrichtte hij het in dit proefschrift beschreven promotieonderzoek, onder begeleiding van Dr. J.A.L. Jeneson en Prof. dr. K. Nicolay. Sinds 2003 is hij werkzaam als post-doc op het laboratorium voor Biophysics and Cell Biology aan het Max Planck Institute of Molecular Physiology te Dortmund.