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Functional properties and $[Ca^{2+}]_i$ metabolism of creatine kinase—KO mice myocardium

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Abstract

One major function of the creatine kinase system is to maintain energy demand of myofibrillar contraction processes. Loss of the CK-system led to adaptations in skeletal muscle. To analyze the impact on myocardial function contractile parameters and intracellular calcium metabolism of transgenic mice lacking mitochondrial CK (ScCKmit$^{-/-}$) alone or both mitochondrial and cytoplasmic ScCK (CK$^{-/-}$) were investigated compared to wild type at various workload conditions using isolated intact muscle fibers. Force development at baseline conditions, force–frequency relationship (60–600/min), and rapid frequency switch (60–600/min) were unaltered in myocardium of transgenic mice compared to wild type. Intracellular calcium metabolism revealed unchanged amplitude of the intracellular calcium transients (ICT), refilling of the sarcoplasmic reticulum (calcium reuptake, post-rest behavior) in the ScCKmit$^{-/-}$ and CK$^{-/-}$ mice. The results demonstrate the effectiveness of myocardial energy-recruiting compensatory mechanisms at baseline as well as under stress conditions in CK depleted myocardium of transgenic mice.

Keywords: Creatine kinase; Transgenic mice; Calcium; Intact fibers; Myocardium; Energy metabolism; Force–frequency relationship; Post-rest behavior

The creatine kinase (CK) enzyme system is an important energy delivering system in muscular (both skeletal and cardiac muscles) and central nervous systems consisting of four different isoenzymes. CK catalyzes the reversible transfer of a high-energy phosphoryl group between ATP and phosphocreatine (PCr) [1]. These isoenzymes are localized in a compartmentalized fashion linking the cellular sites of ATP production and utilization. MM-CK, the most abundant muscle isoform, is a structural protein of the myofibrillar M-band. ScCKmit, the second most abundant isoform, is found on the outer surface of the inner mitochondrial membrane forming a functional compartment with porin and adenine nucleotide translocase [2]. The PCr–CK system has been generally regarded as a high-energy buffer system, which meets increased energetic requirements during periods of mismatched energy production and consumption. The functional importance of the CK system for the heart can be estimated by the well-established changes of nearly all components of this system in disease states such as human heart failure as well as experimental models of heart failure [3,4]. With the help of current transgenic techniques new light has already been shed on the importance of the different CK isoenzymes for heart and skeletal muscles.

Skeletal muscle of mice lacking the M-subunit of CK (ScCKmit$^{-/-}$) demonstrates a transient impairment in contractile function undergoing rapid high-stimulation conditions (burst activity) [5]. In contrast, in cardiac muscle contractile performance of isolated perfused hearts was unchanged for low to moderate workloads in CK$^{-/-}$ and ScCKmit$^{-/-}$ mice [6,7]. However, little is known regarding the myocardial response upon rapid workload changes comparable to burst stimulation of skeletal muscle. Moreover, although a close link between energetics and cardiac metabolism in skeletal

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muscle in CK deficient mice has been shown, measurements regarding intracellular calcium handling over the broad range of cardiac stimulation frequencies are missing. In order to be able to detect immediate changes of contractile response on a beat-to-beat basis the model of isolated, intact myocardial fibers was chosen. This model allows the precise simultaneous measurement of contractile performance and intracellular calcium handling at optimal muscle length at varying stimulation frequencies in order to illuminate the full bright of contractile answers at different, predefined workload situations.

Methods

Animals

ScCKmit$^{-/-}$ mice (20–30 weeks of age) were obtained by gene targeting (B. Wieringa/University of Nijmegen, The Netherlands) as previously reported [8]. There was no difference between WT, ScCKmit$^{-/-}$, and CK$^{-/-}$ mice regarding heart weight or heart weight/body weight ratios. The genotype of each mouse was confirmed by measuring the isoenzymes of CK present using a Helena Cardio-Rep CK isoenzyme analyzer (Helena Diagnostika GmbH).

Isolated heart muscle preparations

After anesthesia of the mice the hearts were quickly removed and perfused with a 4 °C cold Krebs–Henseleit solution (KHS) containing 30 mmol of 2,3 butanedione-monoxime (BDM) to achieve optimal storage conditions. Papillary muscle strips were dissected out of the right ventricle (4×0.5 mm) and stored in the cold KHS/BDM solution until experiments in order to minimize cutting injury [9,10]. The fibers were then mounted in a special setup (Scientific Instruments, Heidelberg, FRG, Fig. 1) between a force transducer and servomotor and constantly perfused with a 37 °C warm, oxygenated KHS-solution [composition in mM/l: NaCl 119.0, KCl 4.6, $NaHCO_3$ 25.0, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.3, glucose 11.0, pH 7.4, and $pO_2 > 500$ mmHg]. After carefully prestretching to optimal length (L_{max}, muscle length at which the maximum twitch amplitude was achieved) electrical stimulation with 1 Hz (15% above threshold) was performed until steady-state conditions were reached.

Experimental protocols

Isometric force contractile analysis. Several mechanical experimental protocols were performed: (1) Force–frequency relationship: the fibers were electrically stimulated with increasing stimulation frequencies (60, 120, 180, 300, 450, and 600/min), developed systolic force was registered at each frequency step. Time from resting tension to peak tension (to-tp) and time from peak tension to 50% and 90% relaxation (tp-$t_{50\%/90\%}$) were analyzed.

Burst activity/rapid switch. In order to analyze the response of heart muscle upon rapid workload change under steady-state conditions stimulation was rapidly switched from 60 to 600 stimuli/min. The first 40 consecutive beats after onset of rapid pacing were analyzed regarding force generation (Fig. 2).

Post-rest behavior. To analyze sarcoplasmic reticulum function post-rest experiments were performed. After continuous stimulation with 60/min or 600/min, respectively, stimulation was switched off for 60 s. The first beat after reinitiating of stimulation was analyzed regarding post-rest force development.

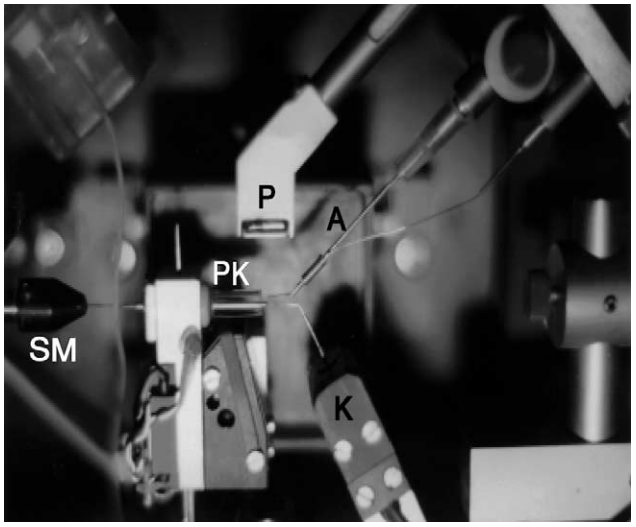

Fig. 1. Experimental set-up: the muscle fibers were fixed between a force-transducer (K) and a servomotor (SM), electrically stimulated, and constantly perfused with an oxygenated Krebs–Henseleit solution in a special perfusion cuvette (PK, A = suction). The emitting fluorescence light was collected in the respective channels of a photomultiplier (P). Simultaneous measurements of force parameters and intracellular calcium transients are therefore possible. For details see Methods.

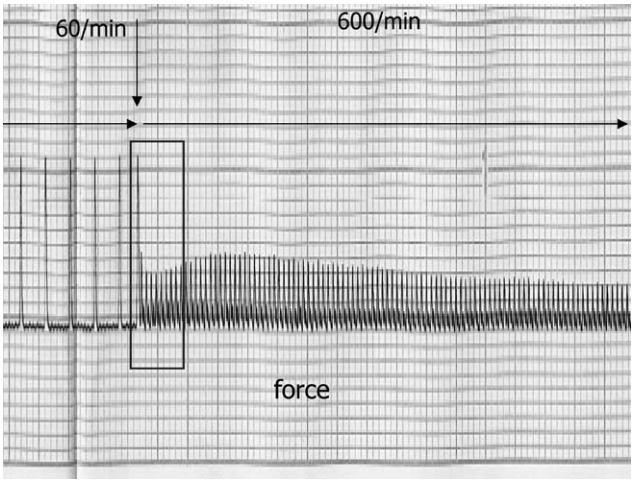

Fig. 2. Original trace of rapid switch experiment: the muscle fiber is stimulated electrically with 60/min. After rapid switch to 600/min the first consecutive force transients were analyzed regarding isometric force development and reaching steady-state conditions.

Intracellular calcium measurements with FURA-2/AM

For analysis of the intracellular calcium transient the muscle fiber was optimally prestretched and electrically stimulated with 1 Hz until steady state conditions are reached (isometric contractions). Thereafter, the fiber was dismounted, rinsed and incubated in a KHS solution containing 5 μmol FURA-2/AM, Cremophor EL (0.5%), and TPEN (*N*,*N*,*N*′, *N*′tetrakis (2-pyridylmethyl) Ethylenediamine) for 4 h as previously described [9]. After incubation the fiber was rinsed with oxygenated KHS-solution and remounted in the experimental setup and stimulated again with 1 Hz for further analysis. Only fibers

reaching at least 80% of the preincubational performance were included in the further analysis.

For simultaneous analysis of the intracellular calcium transient (ICT) together with mechanical parameters the fibers were illuminated within a special perfusion cuvette which allows illumination of the fibers with alternating wavelengths of 340 and 380 nm [11]. The emitted fluorescence light was collected at 510 nm in a photomultiplier. After electronic background fluorescence subtraction the ratio of both fluorescence signals was monitored continuously [10]. The amplitude of the calcium transient and time course for calcium reuptake (time from peak systolic calcium to 50%/90% decrease) were calculated at the stimulation frequencies of the force–frequency relationship (60–600/min).

Statistical analysis

All data are expressed as means ± SEM. Paired and unpaired Student's t tests as appropriate were used to compare ScCKmit$^{-/-}$, CK$^{-/-}$, and wild-type hearts. Statistical analyses were performed with the use of Statview (Brainpower, Calabasas, CA) and values of $p < 0.05$ were considered statistically significant.

Results

Force–frequency relationship

Systolic force decreased in muscle fibers of wild type as well as of CK deficient hearts from 60/min (WT 4.9 ± 0.84 mN/mm^2, ScCKmit$^{-/-}$ 4.09 ± 0.49 mN/mm^2, and CK$^{-/-}$ 4.43 ± 0.92 mN/mm^2) to 600/min (WT 1.73 ± 0.48 mN/mm^2, ScCKmit$^{-/-}$ 1.94 ± 0.32 mN/mm^2, and CK$^{-/-}$ 1.87 ± 0.56 mN/mm^2, $p < 0.01$ vs. 60/min for all groups, n.s. between groups). Force recovered to baseline values after stimulation was returned to 60/min again. However, no significant differences between the groups of wild type, ScCKmit$^{-/-}$, and CK$^{-/-}$ mice at any frequency step could be observed (Fig. 3).

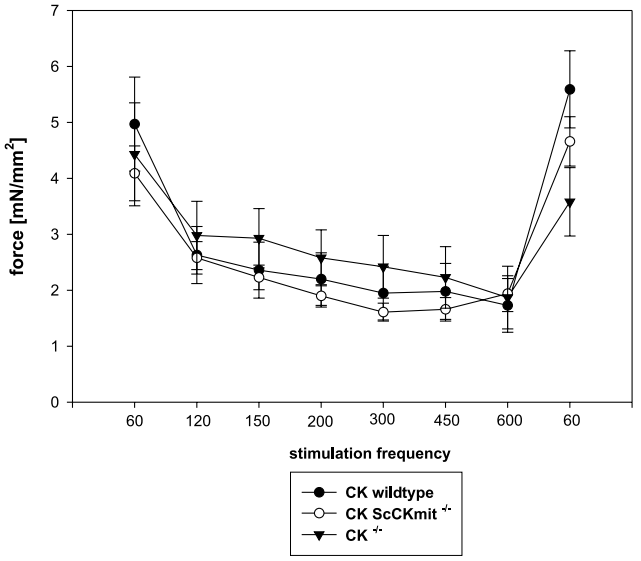

Fig. 3. Force–frequency relationship (60–600/min) of heart muscle fibers derived from CK wild type ($n = 14$), ScCKmit$^{-/-}$ ($n = 7$), and CK$^{-/-}$ ($n = 9$) mice. There are no significant differences between the groups over the entire frequency range detectable.

To-tp, tp-t50% relax, tp-t90% relax

Force time constants were estimated under isometric conditions: time from resting tension to peak tension (t0-tp) was similar in all groups (60/min: WT 115.2 ± 1.4 ms, ScCKmit$^{-/-}$ 109 ± 1.4 ms, and CK$^{-/-}$ 114 ± 2.0 ms; 450/min: WT 42.1 ± 0.9 ms, ScCKmit$^{-/-}$ 43.4 ± 1.1 ms, and CK$^{-/-}$ 37.9 ± 4.2 ms). Furthermore, relaxation parameters (time from peak tension to 50% (tp-$t_{50\%}$) or 90% (tp-$t_{90\%}$) relaxation) were not different between groups. This was found at low- and high-pacing frequencies (Fig. 7)

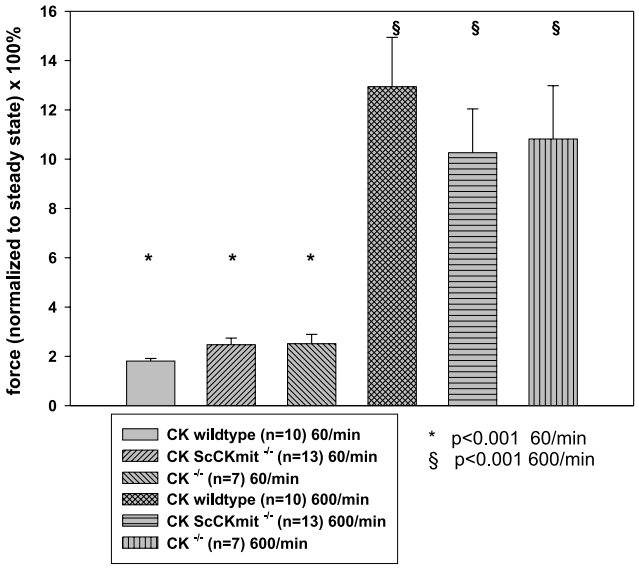

Fig. 4. Post-rest behavior after 10 s cessation of stimulation at 60/min and 600/min. All 3 groups present similar characteristic post-rest behavior at both stimulation frequencies.

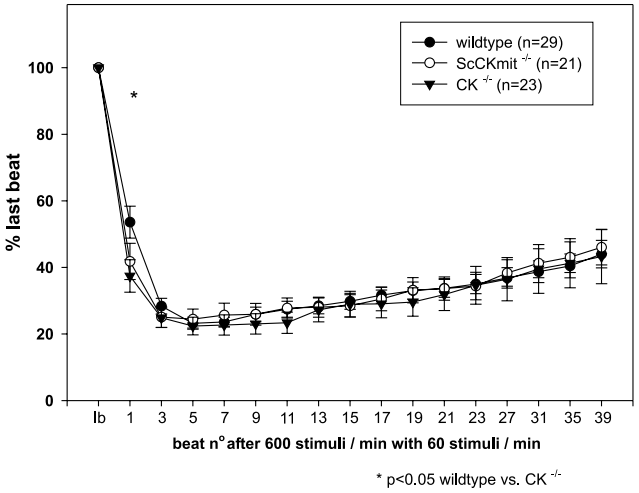

Fig. 5. Rapid switch experiments: the first 40 beats after rapid stimulation switch from 60/min to 600/min were analyzed. The first beat after frequency switch was significantly elevated in wild-type mice compared to CK$^{-/-}$ mice. Already after the third beat all groups presented similar contractile behavior.

Post-rest potentiation (60/600/min)

Isometric force development after reinitiating of stimulation after 10 s without stimulation at 60/min and 600/min increased developed force significantly (post-rest potentiation) in all groups ($p < 0.001$) but without significant differences between the groups. At 60 stimuli/min a 2-fold and at 600/min an over 10-fold increase of the developed force was present in all groups ($p < 0.001$ vs. baseline, Fig. 4).

Burst-activity/rapid switch

The first twitch after rapidly switching the stimulation frequency from 60/min to 600/min was significantly lower in $CK^{-/-}$ mice compared to wild-type mice (Fig. 5 $p < 0.05$). However, already after the second beat after initiation of rapid pacing no significant differences between the isolated muscle strips of wild type, $ScCKmit^{-/-}$, and $CK^{-/-}$ hearts were observed.

Intracellular calcium $[Ca^{2+}]_i$ amplitude and removal kinetics

The amplitude of the intracellular calcium transient significantly decreased with increasing stimulation frequencies. However, there were no significant differences at any stimulation frequency between all groups over the frequency range from 60/min to 600/min (Fig. 6). $[Ca^{2+}]_i$ removal, as indicated by the time from peak $[Ca^{2+}]_i$ to 50% and 90% decline of the calcium transient, which

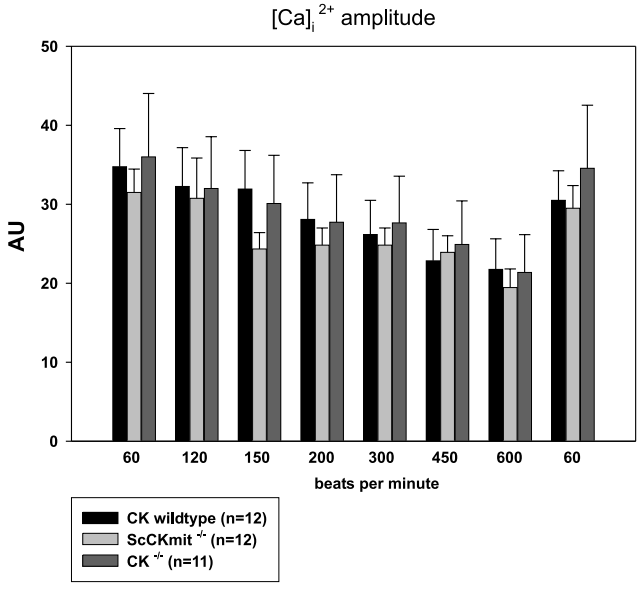

Fig. 6. Amplitude of the intracellular calcium transient (ICT) of intact myocardial fibers of wild type ($n = 12$), $ScCKmit^{-/-}$ ($n = 12$), and $CK^{-/-}$ ($n = 11$) mice as measured with the FURA-2 ratio method. At increasing stimulation frequencies the amplitude of the ICT decreased and returned to initial values after returning to initial stimulation frequency (60/min). AU, arbitrary units.

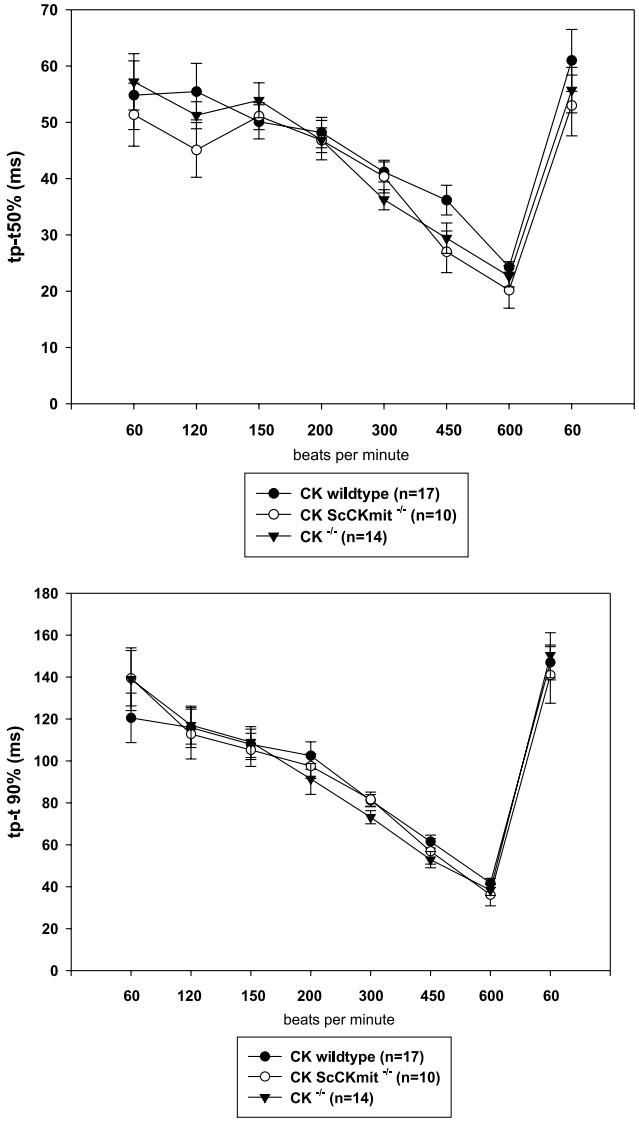

Fig. 7. Time from peak $[Ca^{2+}]_i$ signal to 50% and 90% calcium transient decrease at various stimulation frequencies (60/min to 600/min) as a parameter of intracellular calcium reuptake behavior.

was accelerated for increasing stimulation frequencies (Fig. 7). With increasing stimulation frequency tp-$t_{50\%}$ and tp-$t_{90\%}$ physiologically shortened. However, calcium reuptake was the same in wild type, $ScCKmit^{-/-}$, and $CK^{-/-}$ mice myocardium for the various stimulation frequencies (Fig. 7).

Discussion

The present study was designed to investigate the impact of loss of creatine kinase function under baseline conditions and after rapid workload changes (stress) in heart muscle strips of CK deficient mice compared to wild type. The muscle fiber setup was chosen to exclude confounding neuro-humoral side-effects and to achieve

exactly comparable conditions of contractile analysis. Furthermore, mechanical parameters and the intracellular calcium metabolism could be simultaneously analyzed. In contrast to skeletal muscle, muscle fibers of the myocardium did not reveal significant changes of contractile characteristics or calcium metabolism in mice deleted of either mitochondrial or both mitochondrial and cytoplasmic creatine kinase. These results indicate that sufficient compensatory mechanisms might be present to maintain normal myocardial contractile performance even under high-performance conditions.

Unchanged contractile behavior

To imitate rapid workload changes—comparable to burst activity in skeletal muscle—a protocol of a rapid stimulation frequency switch (RSFS) from 60 to 600 stimuli/min was performed. In contrast to the results of van Deursen and coworkers in skeletal muscle [5] isometric twitch force in myocardial fibers of $CK^{-/-}$ mice was only different at the first beat after RSFS with lower amplitude. Already at the next beat isometric myocardial fiber performance was indistinguishable between all groups within the next 38 beats after RSFS (Fig. 5). To analyze frequency dependent contractile response force frequency relationship (FFR) experiments were performed. With similar force development FFR was negative in fibers of wild type, $ScCKmit^{-/-}$, and $CK^{-/-}$ hearts, as indicated with a decline in force development for increasing stimulation frequencies (Fig. 3). The results are in line with data of Meyer et al. [12] presenting a negative FFR in mice myocardial muscle fibers of wild type and SERCA2 overexpressing mice. Furthermore the fiber diameter of less than 0.5 mm guarantees adequate oxygenation even of the core of the fiber. Hypoxia can therefore be ruled out as a potential source for the negative FFR. Finally contraction and relaxation constants were similar in all groups, indicating the surprising non-influence of a congenitally deleted CK system on myocardial baseline contractility.

Intracellular calcium metabolism

Intracellular calcium handling was altered in skeletal muscle of $CK^{-/-}$ mice where the calcium removal rate and the amount of calcium released per transient were gradually reduced in CK deficient mice compared to wild type [13]. Refilling kinetics of the sarcoplasmic reticulum (SR)'s $[Ca^{2+}]_i$ depends on the CK/phosphocreatine circuit in skeletal muscle. A prolongation of the calcium reuptake would be damasked by a prolongation of the time needed for the intracellular calcium transient (ICT) to return to baseline as calculated by tp-$t_{50\%/90\%}$. However, intracellular calcium kinetics, as measured with FURA-2/AM, was similar in wild type, $ScCKmit^{-/-}$, and $CK^{-/-}$ mice: calcium amplitude over the frequency range (60–600/min) and calcium reuptake, as estimated by tp-$t_{50\%/90\%}$ of the intracellular calcium transient (ICT), did not reveal significant changes (Figs. 6 and 7). However, no quantification of the calcium signal was performed. Differences between the groups regarding absolute diastolic and peak systolic calcium levels are unlikely, but cannot be finally ruled out. However, the unchanged calcium characteristics of both $ScCKmit^{-/-}$ and $CK^{-/-}$ mice are in line with the unchanged contractile behavior of the myocardial muscle fibers as seen in these groups in the present study.

Possible compensatory mechanisms

Excluding the first beat after rapid frequency switch the contractile baseline characteristics, force–frequency relationship, post-rest potentiation behavior, and the intracellular calcium metabolism were not significantly altered between the groups lacking partly or completely creatine kinase isoenzymes. Although creatine kinase is necessary to match energy demand and support, compensatory mechanisms might occur early [14] and must be present to substitute for the missing energy support provided by the CK shuttle: preservation of the CK shuttle and energetic properties are achievable by relocation of cytosolic MM–CK to the outer mitochondrial membrane, where it is coupled to oxidative phosphorylation by close proximity to porin, and the adenin nucleotide translocase [15]. Kaasik and coworkers [16] could demonstrate that in CK deficient mice ultrastructural changes occur. Abundant mitochondria form bulk regions and fill all the space between myofibrils. Furthermore sarcomere structure was altered with decreased A-bands in CK deficient mice. Overall, a direct interaction of mitochondria and the main energy consuming compartment, the sarcoplasmic reticulum, and the myofibrils was postulated [16–19]. Furthermore glycolysis can replace the complete loss of the CK system in fueling the SERCA ATPase [20–22]. This might explain the unchanged Ca^{2+} extrusion characteristics as an indirect indicator of SR function seen in myocardial fibers from both $CK^{-/-}$ and $ScCKmit^{-/-}$ mice. However, the loss of CK activity becomes more energetically "costly" in terms of high-energy phosphate use, accumulation of ADP, and decrease in the free energy released from ATP hydrolysis [6,8]. The compensatory resiliency of loss of function and redirection of flux distribution in the metabolic network for cellular energy in $CK^{-/-}$ mice are made visible in the unchanged contractility and intracellular calcium handling of the heart muscle fibers.

Conclusion

Although the creatine kinase system plays a central role in energy delivering processes, complete congenital deletion of the CK-system seems to be correlated with

sufficient compensatory mechanisms to keep the energy supply adequate under baseline conditions as well as under rapid workload changes in myocardium of either ScCKmit$^{-/-}$ or CK$^{-/-}$ deficient mice. These compensatory mechanisms are even more pronounced in myocardial tissue compared to skeletal muscle as—compared to previous results achieved in skeletal muscle—no differences in mechanical parameters, rapid stimulation frequency—switch, and intracellular calcium characteristics are present.

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