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Chapter 11

The phosphocreatine circuit and creatine supplementation, both come of age!

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Summary

Creatine kinase (CK) isoenzymes, specifically located at places of energy demand and energy production, are linked by a phosphocreatine/creatine (PCr/CRT) circuit, found in cells with intermittently high energy demands. Cytosolic CKs, in close conjunction with Ca^{2+}-pumps, play a crucial role for the energetics of Ca^{2+}-homeostasis. Mitochondrial CK (Mi-CK), a cuboidal-shaped octamer with a central channel, binds and cross-links mitochondrial membranes and forms a functionally coupled microcompartment with porin and adenine nucleotide translocase for vectorial export of PCr into the cytosol. The CK system is regulated by adenosine monophosphate-activated protein kinase (AMPK) via PCr/CRT and adenosine triphosphate/adenosine monophosphate (ATP/AMP) ratios. Mi-CK stabilizes and cross-links cristae-membranes or inner/outer membranes to form parallel membrane stacks and, if overexpressed due to CRT-depletion or cellular energy stress, forms those crystalline intramitochondrial inclusions often seen as hallmarks in mitochondrial cytopathy patients. Mi-CK is a prime target for free radical damage by peroxynitrite. Mi-CK octamers, together with CK substrates, have a marked stabilizing and protective effect against mitochondrial permeability transition pore opening, thus providing a rationale for CRT supplementation of patients with neuromuscular and neurodegenerative diseases. In addition to the well documented improvement of high-intensity intermittent exercise performance after CRT supplementation, recent results seem to indicate that CRT supplementation may also favorably affect long-endurance exercise. Chronic CRT ingestion at high dosage, however, was shown to down-regulate the expression and/or accumulation of CRT transporter polypeptides in muscle. These findings call for an adjustment of the CRT supplementation schedules widely used by many athletes. An intermission of one month, after three months of CRT intake, may thus be advisable.

Discussion

The creatine kinase/phosphocreatine circuit

The enzyme CK, catalyzing the reversible transfer of the N-phosphoryl group from phosphocreatine (PCr) to ADP to regenerate ATP, plays a key role in the energy homeostasis of

cells with intermittently high, fluctuating energy requirements, e.g. skeletal and cardiac muscle, neurons, photoreceptors, spermatozoa and electrocytes. Cytosolic CK isoenzyme(s) (MM-CK, MB-CK and BB-CK) are always co-expressed in a tissue-specific fashion together with a mitochondrial isoform. Using biochemical fractionation and *in situ* localization, we were able to show that the CK isoenzymes, earlier considered to be strictly soluble, are in fact compartmentalized subcellularly and coupled functionally and/or structurally either to sites of energy production (glycolysis and mitochondria) or energy consumption [cellular adenosine triphosphatases (ATPases), such as the acto-myosin ATPase and SR-Ca^{2+}-ATPase]. Thus they form an intricate, highly regulated energy distribution network—the so-called PCr-circuit or PCr-shuttle (Figure 11.1; for review see reference 1 and the special volumes of *Mol. Cell Biochem.* **133/134**, 1994, and **184**, 1998).

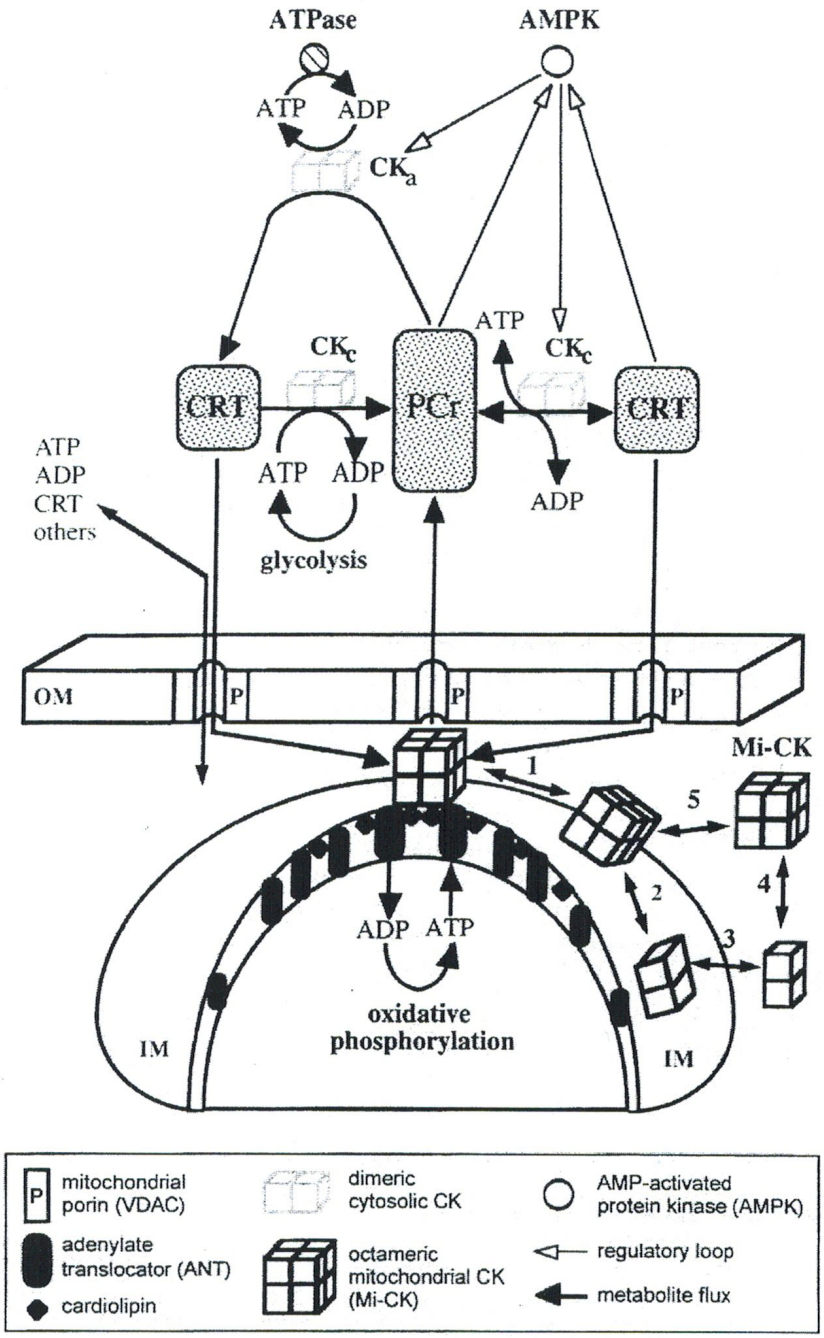

Figure 11.1 (facing) The PCr-circuit: a temporal and spatial energy buffering network and regulatory system for energy metabolism in cells with intermittently high energy requirements.

Upper, cytosolic side: the bulk of soluble, cytosolic CK (CK_c) equilibrates global ATP/ADP and PCr/CRT ratios by its equilibrium reaction (depicted in the middle right of the figure). In skeletal muscle at rest, these metabolite levels are approximately 3–5 mM/10–20 μM and 20–40 mM/10–15 mM, respectively [1, 22, 47]. One of the main functions of CK_c is to keep the concentration of free global ADP very low and thus to maintain global ATP remarkably stable also during cell activation. This part of the PCr-circuit model represents the classic textbook function of CK as a temporal energy buffer, being backed up by adenylate kinase as a second safeguard against declining ATP and rising ADP levels. Some of the cytosolic CK_c is functionally coupled to glycolysis and, during periods of anaerobic work-output and recovery, preferentially accepts glycolytic ATP to replenish the very large PCr pool (ATP from glycolysis, depicted in the middle left of the figure). Additionally, however, some fractions of cytosolic CK are very specifically associated (CK_a) with ATP requiring processes at sites of energy consumption. For example, CK_a is associated with the contractile apparatus and the sarcoplasmic reticulum, where it forms functionally coupled microcompartments with the acto-myosin ATPase and the SR-Ca^{2+}-ATPase, respectively, or with other ATP requiring processes, like the Na^+/K^+-ATPase, etc. (see top of figure). There, ATP is directly regenerated in situ *by CK_a via PCr, thus keeping local ATP/ADP ratios very high in the immediate vicinity of these ATPases.*

CK is phosphorylated and down-regulated in its activity by AMP-dependent protein kinase (AMPK, top right), which itself is the first enzyme that has been found to be regulated by the PCr/CRT ratio, that is, AMPK is activated by high CRT versus PCr levels [20].

Lower mitochondrial side: mitochondrial Mi-CK is bound to the outer side of the inner mitochondrial membrane (IM) and localized along the cristae membranes, as well as at mitochondrial contact sites, where IM and OM are in close vicinity [48]. At these sites, Mi-CK octamers are forming microcompartments with porin (P) and adenine nucleotide translocase (ANT) for energy transfer from ATP to CRT, followed by vectorial transport of PCr into the cytosol. ATP generated by oxidative phosphorylation is preferentially accepted by Mi-CK octamers, transphosphorylated onto CRT, which is entering through mitochondrial porin (VDAC) to give PCr which then is exported into the cytosol. Thus, under high work-load, PCr would be shuttled from mitochondria to sites of energy consumption (ATPases, top of figure), where it is used by CK_a to regenerate ATP locally in situ *to fuel these ATP-requiring processes and to keep local ATP/ADP ratios very high. Creatine would diffuse back to the mitochondria to be recharged again. This part of the model represents the spatial buffering function of the PCr-circuit. The specifically localized CK isoenzymes at sites of energy consumption and energy production are connected via PCr and CRT as mediators, generating metabolic waves and dampening oscillations of metabolites [22, 46].*

The dynamic recruitment of either free or membrane-bound Mi-CK octamers (double-arrows 5 or 1, respectively), possibly depending on the metabolic state of the mitochondria, the dynamic octamer/dimer equilibrium of Mi-CK (double arrows 2 and 4), as well as octamerization of Mi-CK dimers bound on the IM (double-arrow 2), all observed in vitro, *are schematically visualized as potential modulatory events for long-term metabolic regulation. The interaction of Mi-CK with porin and complex formation of the enzyme with ANT, most likely facilitated by cardiolipin associated with ANT, are also illustrated. Under the conditions expected to prevail in the mitochondrial intermembrane space, however, the equilibria of these reactions, as observed in vitro, would clearly favour the membrane-bound octamer [21, 25]. However, since the formation of contact sites and the establishment of protein complexes are thought to be rather dynamic, an on/off recruitment of Mi-CK octamer into contact sites could easily be envisaged. Finally, these events that are heavily influenced by the exquisite sensitivity of Mi-CK towards peroxynitrite and other ROS [26], may be relevant also for the control of the permeability transition pore [39–41, 45].*

This non-equilibrium energy transport model has been challenged, based upon global ^{31}P-nuclear magnetic resonance (^{31}P-NMR) experiments, measuring CK-mediated flux in muscles at different work-loads [2, 3]. The conclusions reached by these authors were: that the CK system is in equilibrium with the substrates, behaving like a solution of well-mixed enzymes; that effects of compartmentation were negligible with respect to total cellular bioenergetics; that thermodynamic characteristics of the cytosol could be predicted as if the CK metabolites were freely mixing in solution. However, based on the organizational principles of sarcomeric muscle, as well as on our findings concerning the highly structured subcellular CK-compartments, this interpretation seemed rather unlikely and thus has been questioned [4]. In support of this, ^{31}P-NMR CK-flux measurements with transgenic mice showing graded reductions of MM-CK expression in their muscles, revealed a strikingly unexpected, 'anomalous' CK-flux behaviour [5]. These results indicate that some flux through CK, presumably bound CK, and possibly also some PCr and/or ATP, is NMR-invisible or otherwise not amenable to this analysis [4, 6]. In the meantime, more evidence from NMR-measurements [7–10], as well as from recent *in vivo* ^{14}[C]CRT-tracer studies [11], is accumulating in favour of compartmentation of the CK system and for the existence of different pools of CK substrates. As a matter of fact, it has now become clear that, in muscle, CRT and PCr molecules do not tumble freely, but display partial orientational ordering, which is in contrast to what is expected for small molecules dissolved in water [7]. Furthermore, ^{31}P-NMR saturation transfer experiments with sea-urchin spermatozoa show that the CK-flux increases by a factor of 10–20 upon sperm activation [12]. These specialized sperm cells derive their energy for motility entirely from fatty oxidation within the single large mitochondrion located just behind the sperm head, from where PCr is diffusing along the 50 μm long sperm tail to fuel the dynein/tubulin ATPase. It is obvious that in these polar, elongated cells, the diffusional limitation of ADP is the key limiting factor with respect to high-energy phosphate provision [13]. Also in support of the PCr-shuttle model, the calculated diffusional flux of ADP in these sperm cells is by 2 and 3 orders of magnitude smaller than those of ATP and PCr, respectively [13].

In conclusion, it becomes obvious that calculations of free cellular [ADP] by using global [ATP] and [PCr], determined by *in vivo* ^{31}P-NMR, together with the CK equilibrium constant, may be valid only in certain limited cases, e.g. in fast twitch glycolytic white muscle fibers, where the buffer function of CK by far prevails the transport function and where the flux through the CK reaction at rest and during high work load are higher by a factor of 100 and 20, respectively, than the total cellular ATPase turnover at these respective states. In cases where the transport function of the CK prevails, e.g. oxidative tissues or in polar cells (sea urchin sperm) with high concentrations of Mi-CK, local ADP and ATP levels, e.g. in the mitochondrial intermembrane space or near CK-ATPase complexes, may differ by orders of magnitude compared to the bulk concentrations calculated from the CK equilibrium constant. Considering the complications of subcellular compartmentation of CK isoenzymes in a cell where, after activation, some CK will work in the forward direction and some in the reverse direction, the interpretation of global CK flux measurements may also represent a rather difficult endeavour.

The importance of creatine kinase for Ca^{2+}-homeostasis and muscle contraction

Transgenic CK(-/-) double knock-out mice show significantly increased relaxation times of their limb muscles, and altered Ca^{2+}-transients in myotubes after stimulation, as well as remarkable remodeling of the contractile apparatus with increased numbers of mitochondria and grossly over-produced tubular SR membranes [14]. The obvious difficulties of these mice with muscle Ca^{2+}-handling, as the main phenotype, is in line with biochemical and functional data showing that some MM-CK is specifically associated with SR membranes [15], where

it is crucial for fueling the energetically highly demanding Ca^{2+}-ATPase [15–17]. Therefore, the most crucial function of the CK-system in muscle is related to the energetics of Ca^{2+}-homeostasis[6].

In addition, some CK is also associated with the myofibril [1]. The domain responsible for the isoenzyme-specific binding of MM-CK to the myofibrillar M-band has been localized by an *in situ* biochemical approach, using heterologously expressed, fluorescently labelled site-directed mutants, as well as M/B-CK chimaeras for diffusion into chemically skinned skeletal muscle fibers [18]. This M-band interaction domain could be narrowed down to two 'charge-clamps', symmetrically organized on an exposed face of each M-CK monomer [80]. Using the same approach to study the weak MM-CK binding to the myofibrillar I-band, observed by *in situ* immunofluorescence localization, we found that MM-CK binding to this sarcomeric region is mediated by some glycolytic enzymes [19].

AMP-activated protein kinase: a ratiometric PCr/CRT energy sensor at last

According to recent findings, AMP-activated protein kinase (AMPK) is able to bind rather tightly to muscle-type MM-CK and phosphorylate the latter to inhibit its activity to a certain extent. AMPK itself is regulated not only by the ATP/AMP ratio, but also by the PCr/CRT ratio [20]. This invalidates the long-held dogma that PCr and CRT are metabolically completely inert compounds. Thus, AMPK, as an energy sensor system, could represent the missing link for regulation of adaptive metabolic changes, e.g. after depletion of CRT levels in skeletal and cardiac muscle. Interestingly enough, both the ablation of the muscle-type CK isoenzymes in transgenic animals [14] or the depletion of CRT, the substrate of the CK reaction, after supplementation with β-phosphorylated guanidinopropionic acid (β-GPA)[50], seem to elicit very similar adaptational effects in skeletal muscle. The activation of AMPK by decreasing PCr/CRT ratios and increasing AMP, as observed during muscle activation at high work-load would lead to progressively stronger inactivation of cytosolic muscle-type MM-CK [20]. This could very well explain the long-standing enigma of why, in muscle, the CK-mediated reaction flux, which can be more than 10-fold to 20-fold higher than the highest ATPase turnover, depending on the muscle type, does not increase with higher workload, but rather tends to decrease instead [78, 79].

Mitochondrial creatine kinase for metabolic channeling of high-energy phosphate compounds

Mitochondrial creatine kinase (Mi-CK) is located in the mitochondrial intermembrane space along the inner membrane, but also at contact sites where inner and outer membranes are in close proximity[7,48]. Mi-CK can directly transphosphorylate intramitochondrially-produced ATP into PCr, which subsequently is exported to the cytosol. A well-documented role of Mi-CK is the functional coupling of mitochondrial CK to oxidative phosphorylation [21, 22], which facilitates the antiport of ATP versus ADP through the inner membrane via the adenine nucleotide translocator (ANT). In addition, a physical interaction of Mi-CK with outer mitochondrial membrane porin (VDAC) has also been demonstrated [23]. The recently solved atomic X-ray structure of octameric Mi-CK [24] is consistent with the proposed energy channeling function of this enzyme. Detailed structure/function analyses concerning the molecular physiology, catalytic site and mechanism, octamer/dimer equilibrium, as well as the interaction of Mi-CK with mitochondrial membranes have been published [21, 25]. The identical top and bottom faces of the octamer contain putative membrane binding motifs likely to be involved in the binding of Mi-CK to mitochondrial membranes. The central 20 Angstroms wide channel of the Mi-CK octamer may be of functional significance for the exchange of energy metabolites between mitochondria and cytosol. If Mi-CK would follow a 'back door'

mechanism by which PCr is to be expelled into the central channel of the Mi-CK octamer, as depicted in hypothetical models (see Figures 6A and 7 in reference 21), vectorial transport of PCr from the mitochondrial matrix into the cytosol could be greatly facilitated.

Exquisite sensitivity of Mi-CK to peroxynitrite: effects on cellular Ca^{2+}-homeostasis and linkage to pathological states

Peroxynitrite ($ONOO^-$, PN), the product of the reaction between nitrogen monoxide (NO) and the superoxide anion O_2^- has been shown to be highly reactive towards Mi-CK [26]. Recently, a mitochondrial NO synthase isoform has been discovered [27]. Thus, mitochondria as a notorious source of O_2^-, especially after ischemia/reperfusion episodes, additionally produce PN internally. We have found that Mi-CK in intact mitochondria is a prime target of inactivation and modification by PN, at concentrations of PN that are much lower than those needed for inactivation of mitochondrial respiratory chain enzymes [26]. The pronounced sensitivity of Mi-CK towards reactive oxygen species (ROS), especially peroxynitrite, may explain the effects seen after perturbation of cellular pro-oxidant/antioxidant balance, e.g. after ischemia/reperfusion. These effects include energy failure, paralleled by elevated ADP levels and chronic calcium overload due to inactivation of the CK system. Perfusion of hearts with NO donors lead to an inhibition of cardiac CK by 65% and a concomitant decrease in heart contractile reserve [28]. Stimulation of inducible NO-synthase (NOS), which is indeed increased *in vivo* in skeletal muscle biopsies from patients with chronic heart failure [29], also leads to an NO-dependent depression of cardiac function [30]. Thus, a correlation between a compromised CK system and energy failure of the heart becomes obvious.

Most recently, we found that PN is also affecting the oligomeric state of Mi-CK. PN-treatment of Mi-CK octamers leads to some dimerisation, whereas treatment of dimeric Mi-CK with the same reagent prevents reoctamerization of Mi-CK dimers in a PN-concentration dependent manner [31]. These findings may explain why, in different models of cardiac infarction, one consistently detects a significantly enhanced proportion of Mi-CK dimers as compared to cases of non-infarcted heart tissue[81].

The results that cytosolic CKs, and therefore also SR-bound MM-CK, which is functionally coupled to the SR-Ca^{2+}-pump [15–17], are also very sensitive to reactive oxygen species (ROS) as well [32, 33], indicate that impairment of the CK system by ROS would severely disturb cellular Ca^{2+}-handling and homeostasis. As a consquence of cellular Ca^{2+}-overload resulting, among other factors, in a break-down of mitochondrial membrane potential, mitochondria may release additional Ca^{2+} into the cytosol [34], thus aggravating the situation even more [35]. The interaction of elevated Ca^{2+}-levels and raise in ROS would then lead to a vicious cycle with progressive inactivation of both Mi-CK and SR-bound MM-CK. Therefore, the destabilization of cellular energetics by chronic exposure to ROS, thought to occur in many neuromuscular diseases [36], may finally lead to apoptosis or cell death, especially in those cells with high mitochondrial activity. Skeletal muscle and cardiac or neuronal cells are ideal candidates as chronically elevated Ca^{2+}-levels or Ca^{2+}-overload has been identified as a major player of cell destruction [36]. A clear link between chronically elevated Ca^{2+}-concentration and a calcineurin-dependent signaling pathway, eventually leading to cardiac hypertrophy and chronic heart failure has been demonstrated very recently [35].

In accordance with the CK/ Ca^{2+}-connection, in brain, the concentration of CK was found to be very high in those cells that display high-frequency Ca^{2+}-spiking, e.g. cerebellar Purkinje neurons, as well as granule and pyramidal cells of the hippocampus [37]. A most recent finding, showing that in neurodegenerative diseases, like Alzheimer's disease, CK enzyme activity is severely reduced and cytosol-membrane partitioning is aberrant [38], also corroborates the imporant role of the CK/PCr-system in the energetics of brain pathology.

Involvement of Mi-CK and CK substrates in mitochondrial permeability transition and early apoptosis

A protein complex containing ANT and mitochondrial porin has recently been described to display the characteristics of the mitochondrial permeability transition pore (MTP) or mega-channel [39]. The physical interaction and functional coupling of Mi-CK with porin and ANT indicates an involvement of Mi-CK in the regulation of MTP, since octameric Mi-CK [1] in this protein complex [23, 39, 40], plus CRT or CRT analogs, can delay MTP [41]. This has been demonstrated by using transgenic mice that express Mi-CK in liver. Since liver cells of wild-type animals do not contain this enzyme, but otherwise are identical, mitochondria of livers serve as an ideal control. Our experiments provided exciting new evidence that Mi-CK is not only involved in mitochondrial energy transfer and shuttling of high-energy phosphate, but may also participate directly in mitochondrial permeability transition (MPT) [41].

The Ca^{2+}-dependent increase of inner membrane permability to ions and solutes is dependent on the transmembrane potential difference, matrix pH, SH-group reactants and is modulated by a variety of effectors. Cyclosoporin-A turned out to be a very potent inhibitor of MPT [42]. Interestingly, CRT or cyclocreatine (cCr) delayed cyclosporin-A-sensitive swelling and inhibited concomitant increase of state 4 respiration of mitochondria from Mi-CK-containing transgenic livers [41]. No comparable effect was seen with control liver mitochondria that do not contain any CK. This novel Mi-CK-related phenomenon deserves further attention since it may shed some new light on the recently observed neuroprotective effects of CRT and its analogs in animals models [43, 44].

In addition, protein complexes, containing octameric Mi-CK, porin and ANT, could be isolated from detergent solubilized rat brain extracts [39, 40]. After reconstitution into malate-loaded lipid vesicles, the presence of octameric Mi-CK prevented Ca^{2+}-induced malate release, which, however, was observed after dimerization of Mi-CK [41]. The fact that highly purified ANT, functionally reconstituted as an ATP/ADP exchange carrier, displayed a Ca^{2+}-dependent release of internal substances, while atractyloside or $HgCl_2$ both induced unspecific pore opening of ANT, indicate that ANT is capable of adopting a pore-like structure under conditions known to induce MPT [45]. Mi-CK was shown to be functionally coupled to ANT [1, 2, 46, 47] and to form complexes with porin and ANT [40]. Therefore, it is obvious that Mi-CK octamers could directly affect this ANT-mediated permeability transiton. Thus, the arrangement of Mi-CK as an energy channeling unit, sandwiched in between porin and ANT, and linking OM and IM together, seems not only important for high-energy phosphate conversion and transport (see Figure 11.1), but the molecule may also act as a protective regulatory component of the permeability transition complex. Depending on the cellular energy state and intracellular $[Ca^{2+}]$, octameric Mi-CK may prevent MTP [48], an early event in the execution of apoptosis [49] in cells with high energy demands, thus sparing the cells from or delaying cell death. On the other hand, dimerization of the Mi-CK octamer may allow the ANT to switch to its MTP-like state [48], eventually leading to apoptosis.

Enhancement of physical performance by creatine supplementation

The CK/PCr system is now recognized as an important metabolic regulator during health and disease. Creatine, synthesized in part by the body, but also ingested by food, especially meat and fish [50], is taken up into cells by a creatine transporter (CreaT)[51]. Creatine supplementation in humans leads to an increase in intracellular CRT and PCr, concomitantly improving anaerobic performance of muscle [52, 53] and speeding up recovery after exhaustive excercise [54–56]. We could show, however, that CRT supplementation may also have beneficial effects for high-intensity, aerobic long-endurance exercise [57]. In a double-blinded placebo-controlled study, 20 highly-trained top athletes were subjected, at 1650 meters above sea level (in Davos, Switzerland), to a series of spiro-ergometric short-term and long-term performance

tests, before and after 10 days of supplementation with 3 × 3.3 g of CRT (PODIUM®) per day. In accordance with earlier studies, short performance and maximal work output were both improved by approximately 30 Watts. In a 1 hour spiro-ergometric test at 85% power output of the individually determined anaerobic threshold, the CRT group was able to perform, after CRT supplementation, at the same level of exercise with a significantly lower heart rate (–8.4 beats/min) than before CRT intake. In this group, lactate levels were lower by 0.48 mM/L and Borg scale numbers by 1.35 points. These effects were not observed in the controls. Ventilation, VO_2 and respiratory quotient (RQ) were basically unchanged, indicating mostly a peripheral effect [57]. The effects of CRT on endurance performance observed here seem to be due to increased efficiency of energy utilization by heart and skeletal muscle and may be related to the involvement of CK in the energetics of Ca^{2+}-homeostasis. As a consequence of CRT supplementation, the elevated cellular PCr level increases the supply of the SR-Ca^{2+}-ATPase with high-energy phosphates via coupled CK and, thus, also increases the efficiency of Ca^{2+}-pumping. During long-endurance exercise, this process consumes a significant proportion of the available bioenergy. In addition, CRT-stimulated respiration and enhanced resynthesis of PCr after CRT ingestion [54] and/or the recently discovered control of AMP-activated protein kinase by the PCr/CRT ratio [20] and its effects on CK and lipid metabolism in general[20] could be important factors leading to the observed improvement of aerobic exercise described above.

Down-regulation of the creatine transporter after chronic creatine ingestion

The CreaT, responsible for the uptake of CRT into a variety of tissues and cells, was detected in rat skeletal and cardiac muscle, cerebellum, forebrain and kidney. Two polypeptides with an apparent Mr of 70 kDa and 55 kDa were always recognized by both of our specific polyclonal antibodies directed against synthetic peptides of either the NH_2-terminus or the COOH-terminus of CreaT, indicating a high degree of homology between the two proteins [51]. In contrast to published data obtained by Northern blot analysis, suggesting a complete absence of CreaT mRNA message in liver, we could clearly detect both CreaT polypeptides also in rat liver and hepatocyte lysates. In support of this, cultured hepatocytes show an endogenous CreaT activity which is antagonized by the CRT analog, β-GPA, a well known inhibitor of CreaT. Glyco-staining of CreaT, enriched by immuno-affinity chromatography, mainly containing both the 70 kDa and 55 kDa bands, showed strong glycosylation of preferentially the upper 70 kDa polypeptide indicating that the latter is a post-translationally modified form of the 55 kDa core protein. HeLa cells transfected with rat CreaT cDNA showed an increase in ^{14}C-creatine uptake, when compared to control cells, that was antagonized by β-GPA. In parallel, an increase in the expression of both the 70 kDa and the 55 kDa polypeptides over endogenous CreaT of controls was noticed on Western blots. Furthermore, we have found that chronic CRT supplementation at high dosage of rats down-regulates, *in vivo*, the expression and/or accumulation of the CreaT in skeletal muscle, but not in brain and heart [58]. This finding may have far reaching consequences with respect to CRT supplementation schedules that are widely used by many athletes. They would call for an intermission of CRT intake after three months with a break of a least one month. However, detailed studies on humans are needed to optimize the CRT supplementation schedules in use with respect to the observed down-regulation of CreaT expression and/or accumulation.

Creatine supplementation as adjuvant therapy for neuromuscular diseases

Creatine seems helpful, not only for athletes to improve physical performance on different levels (see above), but it is also emerging as a therapeutic aid for neuromuscular and neurodegenerative diseases. In some of these diseases, especially in mitochondrial myopathies,

a compensatory over-expression of Mi-CK, due to cellular energy deficit, can lead to the formation of pathological intramitochondrial crystalline Mi-CK inclusions [59], that, at least in the β-GPA-animal model, disappear completely upon administration of CRT [60].

A protective effect of CRT on neuronal function, especially during hypoxia or anoxia, was first described some years ago on brain slices [61, 62]. Only recently, encouraged by the success of CRT supplementation for improvement of muscle performance in humans, have CRT and analogs attracted new interest for brain metabolism [63–65]. In animal models, the CRT analog, β-GPA, was shown to remarkably protect the brain of mice from hypoxic damage and seizures *in vivo* [64] and significant neuroprotective effects of CRT and cCr have been described in an animal model of Huntington's disease [44], as well as for Parkinsonism [66]. Creatine and cCr afforded significant protection against malonate, as well as 3-nitropropionic acid (3-NP) lesions and ROS generation in the brain. The observed neuroprotective effects would be fully in line with the high expression levels and localization of CK isoenzymes found in brain, both regionally [37] and on a cellular level [67], as well as functionally in the adult brain [68, 69] or during brain development and maturation [70].

The above neuroprotective effects are paralleled also with astonishing findings in transgenic mice expressing BB-CK in liver, which normally is devoid of CK activity. Livers of such mice become highly resistant to hypoxia [71] and liver toxins [72]. In addition, CK and CRT, improving the intracellular phosphorylation potential of these transgenic livers, confer protection of ATP levels and stabilization of pH during a fructose load [73]. Most recently, CRT supplementation of dystrophic muscle cells from mdx mice was shown to result in a marked cell protection, after a challenge by either hypo-osmotic swelling or high extracellular Ca^{2+}, against chronically elevated calcium levels seen in untreated control cells [74]. Promising preliminary results and favorable subjective feed-back responses with patients suffering from different neuromuscular diseases [75] have stimulated controlled double-blinded clinical studies. Thus, the validity of CRT supplementation as a possible adjuvant therapy for neuromuscular and neurodegenerative diseases is currently being tested.

A bright future can be foreseen for CRT as a nutritional supplement, and a plethora of medical applications for CRT supplementation as an adjuvant therapy wait for serious clinical trials. Finally, for some cases, CRT and its analogs will be used in the future for full-fledged pharmaceutical intervention, e.g. for treating inborn errors of CRT metabolism [76] or for anti-cancer therapy [77].

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References

1. Wallimann, T., Wyss, M., Brdiczka, D., Nicolay, K. and Eppenberger, H. M. (1992): Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'PCr-circuit' for cellular energy homeostasis. *Biochem. J.* **281**, 21–40.
2. McFarland, E. W., Kushmerick, M. J. and Moerland, T. (1994): Activity of creatine kinase in a contracting mammalian muscle of uniform fiber type. *Biophy. J.* **67**, 1912–1924.
3. Wiseman, R. W. and Kushmerick, M. (1995): Creatine kinase equilibrium follows solution thermodynamics in skeletal muscle: ^{31}P-NMR studies using creatine analogs. *J. Biol. Chem.* **270**, 12428–12438.
4. Wallimann, T. (1994): ^{31}P-NMR-measured creatine kinase reaction flux in muscle: a CAVEAT! *J. Muscle Res. Cell Motil.* **17**, 177–181.
5. VanDeursen, J., Ruitenbeek, W., Heerschap, A., Jap, P., terLaak, H. and Wieringa, B. (1994): Creatine kinase in skeletal muscle energy metabolism: a study of mouse mutants with graded reduction in muscle CK expression. *Proc. Natl. Acad. Sci. USA* **91**, 9091–9095.

6. Wallimann, T. (1994): Dissecting the role of creatine kinase. *Current Biology* **1**, 42–46.

7. Kreis, R., Koster, M., Kamber, M., Hoppeler, H. and Boesch, C. (1997): Peak assignment in localized 1H MR spectra of human muscle based on oral creatine supplementation. *Magn. Res. in Med.* **37**, 159–163.

8. LeRumeur, E., LeTallec, N., Kernec, F. and deCertaines, J. D. (1997): Kinetics of ATP to ADP β-phosphoryl conversion in contracting skeletal muscle by *in vivo* ^{31}P-NMR magnetization transfer. *NMR in Biomed.* **10**, 67–72.

9. Ntziachristos, V., Kreis, R., Boesch, C. and Quistorff, B. (1997): Dipolar resonance frequency shifts in 1H MR spectra of skeletal muscle: confirmation in rats at 4.7 T *in vivo* and observation of changes postmortem. *Magn. Reson. Med.* **38**, 33–39.

10. Williams, J. P. and Headrick, J. P. (1996): Differences in nucleotide compartmentation and energy state in isolated and *in situ* rat heart: assessment by ^{31}P-NMR spectroscopy. *Biochim. Biophys. Acta* **1276**, 71–79.

11. Hochachka, P. W. and Mossey, M. K. (1998): Does muscle creatine phosphokinase have access to the total pool of phosphocreatine plus creatine? *Am. J. Physiol.* **274**, R868–R872.

12. VanDorsten, F., Wyss, M., Wallimann, T. and Nicolay, K. (1997): Activation of sea urchin sperm motility is accompanied by an increase in the creatine kinase exchange flux. *Biochem. J.* **325**, 411–416.

13. Kaldis, P., Kamp, G., Piendl, T. and Wallimann, T. (1997): Functions of creatine kinase isoenzymes in spermatozoa. *Adv. in Develop. Biology* **5**, 275–312.

14. Steeghs, K., Benders, A., Oerlemans, F., deHaan, A., Heerschap, A., Ruitenbeek, W., Jost, C., van Deursen, J., Peryman, B., Pette, D., Brückwilder, M., Koudijs, J., Jap, P., Veerkamp, J. and Wieringa, B. (1997): Altered Ca2+-response in muscles with combined mitochondrial and cytosolic creatine kinase deficiencies. *Cell* **89**, 93–103.

15. Rossi, A. M., Eppenberger, H. M., Volpe, P., Cotrufo, R. and Wallimann, T. (1990): Muscle type MM-creatine kinase is specifically bound to sarcoplasmic reticulum and can support Ca^{2+}-uptake and regulate local ATP/ADP ratios. *J. Biol. Chem.* **265**, 5258–5266.

16. Korge, P. and Campbell, K. B. (1994): Local ATP regeneration is important for sarcoplasmic reticulum Ca^{2+}-pump function. *Am. J. Physiol.* **267**, C357–C366.

17. Minajeva, A., Ventura-Calapier, R. and Veksler, V. (1996): Ca^{2+}-uptake by cardiac sarcoplasmic reticulum ATPase *in situ* strongly depends on bound creatine kinase. *Pflügers Arch.* **432**, 904–912.

18. Stolz M. and Wallimann, T. (1998): Myofibrillar interaction of cytosolic creatine kinase (CK) isoenzymes: allocation of N-terminal binding epitope in MM-CK and BB-CK. *J. Cell Sci.* **111**, 1207–1216.

19. Kraft, T., Nier, V., Brenner, B. and Wallimann, T. (1996): Binding of creatine kinase to the I-band of skinned skeletal muscle fibers is mediated by glycolytic enzymes: an *in situ* biochemical approach. *Biophys. J.* **70**, A292.

20. Ponticos, M., Lu, Q. L., Morgan, J. E., Hardie, D. G., Partridge, T. A. and Carling, D. (1998): Dual regulation of AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J.* **17**, 1688–1699.

21. Schlattner, U., Forstner, M., Eder, M., Stachowiak, O., Fritz-Wolf, K. and Wallimann, T. (1998): Functional aspects of the X-ray structure of mitochondrial creatine kinase: a molecular physiology approach. *Mol. Cell Biochem.* **184**, 125–140.

22. Wyss, M., Smeitink, J., Wevers, R. and Wallimann, T. (1992): Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism. *Biochim. Biophys. Acta* **1102**, 119–166.

23. Brdiczka, D., Kaldis, P. and Wallimann, T. (1994): *In vitro* complex formation between the octamer of mitochondrial creatine kinase and porin. *J. Biol. Chem.* **269**, 27640–27644.

24. Fritz-Wolf, K., Schnyder, T., Wallimann, T. and Kabsch, W. (1996): Structure of mitochondrial creatine kinase. *Nature* **381**, 341–345.

25. Stachowiak, O., Schlattner, U., Dolder, M. and Wallimann, T. (1998): Oligomeric state and membrane binding behaviour of creatine kinase isoenzymes: implications for cellular function and mitochondrial structure. *Mol. Cell Biochem.* **184**, 141–151.

26. Stachowiak, O., Dolder, M., Wallimann, T. and Richter, C. (1998): Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. *J. Biol. Chem.* **273**, 16694–16699.

27. Ghafourifar, P. and Richter, C. (1997): Nitric oxide synthase activity in mitochondria. *FEBS Lett.* **418**, 291–296.

28. Gross, W. L., Bak, M. I., Ingwall, J. S., Arstall, M. A., Smith, T. W., Balligand, J. L. and Kelly, R. A. (1996): Nitric oxide inhibits creatine kinase and regulates rat heart contractile reserve. *Proc. Natl. Acad. Sci. USA* **93**, 5604–5609.

29. Adams, V., Yu, J., Möbius-Winkler, S., Linke, A., Weigl, C., Hilbrich, L., Schuler, G. and Hambrecht, R. (1997): Increased inducible nitric oxide synthase in skeletal muscle biopsies from patients with chronic heart failure. *Biochem. Mol. Medicine* **61**, 152–160.

30. Joe, E. K., Schussheim, A. E., Longrois, D., Maki, T., Kelly, R. A., Smith, T. W. and Balligand, J. L. (1998): Regulation of cardiac myocyte contractile function by inducible nitric oxide synthase (iNOS): mechanisms of contractile depression by nitric oxide. *J. Mol. Cell Cardiol.* **30**, 303–315.

31. Wendt, S., Stachowiak, O., Dolder, M., Schlattner, U. and Wallimann, T. (1998): Effects of peroxynitrite on creatine kinase: implications for Ca^{2+}-handling and apoptosis. 5th Internatl. Symp. on Guanidino Compounds in Biology and Medicine (Yokohama, Japan, Sept. 2–3, 1998), Abstract S3-7, p. 63.

32. Mekhfi, H., Veksler, V., Mateo, P., Maupoil, V., Rochette, L. and Ventura-Clapier, R. (1996): Creatine kinase is the main target of reactive oxygen species in cardiac myofibrils. *Circ. Res.* **78**, 1016–1027.

33. Konorev, E. A., Hogg, N. and Kalyanaraman, B. (1998): Rapid and irreversible inhibition of creatine kinase by peroxynitrite. *FEBS Lett.* **427**, 171–174.

34. Dykens, J. A. (1994): Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca^{2+} and Na^{+}: implications for neurodegeneration. *J. Neurochem.* **63**, 584–591.

35. Molkentin, J. D., Lu, J. R., Antos, C. L., Markham, B., Richardson, J., Robbins, J., Grant, S. R. and Olson, E. N. (1998): A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* **93**, 215–228.

36. Mattson, M. P. (1992): Calcium as sculptor and destroyer of neural circuitry. *Exp. Gerontol.* 27, 29–49.

37. Kaldis, P., Hemmer, W., Zanolla, E., Holtzman, D. and Wallimann, T. (1996): Hot spots of creatine kinase localization in brain: cerebellum, hippocampus and choroid plexus. *Dev. Neurosci.* **18**, 542–554.

38. David, S., Shoemaker, M. and Haley, B. E. (1998): Abnormal properties of creatine kinase in Alzheimer's disease brain: correlation of reduced enzyme activity and active site photolabeling with aberrant cytosol-membrane partitioning. *Mol. Brain Res.* **54**, 276–287.

39. Beutner, G., Rück, A., Riede, B., Welte, W. and Brdiczka, D. (1996). Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. *FEBS Lett.* **396**, 189–195.

40. Beutner, G., Rück, A., Riede, B. and Brdiczka, D. (1998): Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. Biochim. Biophys. Acta **1368**, 7–18.

41. O' Gorman, E., Beutner, G., Dolder, M., Koretsky, A. P., Brdiczka, D. and Wallimann, T. (1997): The role of creatine kinase in inhibition of mitochondrial permeability transition. *FEBS Lett.* **414**, 253–257.

42. Crompton, M., Ellinger, H. and Costi, A. (1988): Inhibition by cyclosporin A of a Ca^{2+}-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. *Biochem. J.* **255**, 357–360.

43. Holtzman, D. and Kekelidze, T. (1998): Guanidino analogs and the brain creatine kinase system. 5th Internatl. Symp. on Guanidino Compounds in Biology and Medicine (Yokohama, Japan, Sept. 2–3, 1998), Abstract SL-1, p. 22.

44. Matthews, R. T., Yang, L., Jenkins, B. G., Ferrante, R. J., Rosen, B. R., Kaddurah-Daouk, R. and Beal, M. F. (1998): Neuroprotective effects of creatine and cyclocreatine in animal models of Huntington's disease. *J. Neurosci.* **18**, 156–163.

45. Rück, A., Dolder, M., Wallimann, T. and Brdiczka, D. (1998): Reconstituted adenine nucleotide translocase forms a channel for small molecules comparable to the mitochondrial permeability transition pore. *FEBS Lett.* **426**, 97–101.

46. Wyss, M. and Wallimann, T. (1992): Metabolite channelling in aerobic energy metabolism. *J. Theor. Biol.* **158**, 129–132.

47. Saks, V. A., Khuchua, Z. A., Vasilyeva, E. V., Belikova, O. Y. and Kuznetsov, A. V. (1994): Metabolic compartmentation and substrate channelling in muscle cells. Role of coupled creatine kinases in *in vivo* regulation of cellular respiration – a synthesis. *Mol. Cell Biochem.* **133/134**, 155192.

48. Brdiczka, D., Beutner, G., Rück, A., Dolder, M. and Wallimann, T. (1998): The molecular structure of mitochondrial contact sites. Their role in regulation of energy metabolism and permeability transition. *BioFactors* **8**, 235–242.

49 Leist, M. and Nicotera, P. (1998): Apoptosis, excitotoxicity and neuropathology. *Exp. Cell Res.* **239**, 183–201.

50. Wyss, M. and Wallimann, T. (1994): Creatine metabolism and the consequences of creatine depletion in muscle. *Mol. Cell Biochem.* **133/134**, 51–66.

51. Guerrero-Ontiveros, L. and Wallimann, T. (1998): Creatine supplementation in health and disease. Effects of chronic creatine ingestion *in vivo*: down-regulation of the expression of creatine transporter isoforms in skeletal muscle. *Mol. Cell Biochem.* **184**, 427–437.

52. Greenhaff, P. L. Casey, A., Short, A. H., Harris, R., Soderlund, K. and Hultman, E. (1993): Influence of oral creatine suplementation on muscle torque during repeated bouts of maximal voluntary exercise in man. *Clin. Sci.* **84**, 565–571.

53. Balsom, P. D., Soderlund, K. and Ekblom, B. (1994): Creatine in humans with special reference to creatine supplementation. *Sports Med.* **18**, 268–280.

54. Greenhaff, P. L., Bodin, K., Soderlund, K. and Hultman, E. (1994): Effect of oral creatine supplementation on skeletal muscle phosphocreatine resynthesis. *Am. J. Physiol.* **266**, E725–E730.

55. Hultman, E., Soderlund, K., Timmons, J. A., Cederblad, G. and Greenhaff, P. L. (1996): Muscle creatine loading in man. *Am. J. Appl. Physiol.* **81**, 232–237.

56. Greenhaff, P. L. (1997): The nutritional biochemistry of creatine. *Nutritional Biochem.* **8**, 610–618.

57. Brönnimann, M., Accola, C., Strub, W., Villiger, B. and Wallimann, T. (1998): Beneficial effect of creatine supplementation for high-intensity endurance performance. 5th Internatl. Symp. on Guanidino Compounds in Biology and Medicine (Yokohama, Japan, Sept. 2–3, 1998), Abstract S1-3, p. 32.

58. Guerrero, L. M., Walzel, B. and Wallimann, T. (1998): Creatine transporter polypeptides are down-regulated by chronic creatine supplementation. 5th Internatl. Symp. on Guanidino Compounds in Biology and Medicine (Yokohama, Japan, Sept. 2-3, 1998), Abstract S1-8, p. 37.

59. Stadhouders, A. M., Jap, P., Winkler, H. P., Eppenberger, H. M. and Wallimann, T. (1994): Mitochondrial creatine kinase: a major constituent of pathological inclusions seen in mitochondrial myopathies. *Proc. Acad. Sci. USA* **91**, 5089–5093.

60. O'Gorman, E., Fuchs, K.-H., Tittmann, P., Gross, H. and Wallimann, T. (1997): Crystalline mitochondrial inclusion bodies isolated from creatine-depleted rat soleus muscle. *J. Cell Sci.* **110**, 1403–1411.

61. Woznicki, D. T. and Walker, J. B. (1980): Utilization of cyclocreatine phosphate, an analogue of creatine phosphate, by mouse brain during ischemia and its sparing action on brain energy reserves. *J. Neurochem.* **35**, 1247–1253.

62. Whittingham, T. S. and Lipton, P. (1981): Cerebral synaptic transmission during anoxia is protected by creatine. *J. Neurochem.* **37**, 1618–1621.

63. Carter, A. J., Müller, E., Pschorn, U. and Stransky, W. (1995): Preincubation with creatine enhances levels of creatine phosphate and prevents anoxic damage in rat hippocampus slices. *J. Neurochem.* **64**, 2691–2699.

64. Holtzman, D., Meyers, R., O'Gorman, E., Khait, I., Wallimann, T., Allred, E. and Jensen, F. (1997): *In vivo* brain phosphocreatine and ATP regulation in mice fed a creatine analog. *Am. J. Physiol.* **272**, C1567–C1577.

65. Wilken, B., Ramires, J. M., Probst, I., Richter, D. W. and Hanefeld, F. (1998): Creatine protects the central respiratory network of mammals under anoxic conditions. *Pediatric Res.* **43**, 8–14.

66. Matthews, R. T., Yang, L. C., Ferrante, R. J., Kowall, N. W., Kaddurah-Daouk, R. and Beal, M. F. (1997): Creatine and cyclocreatine protect against cell death in two animal models of neurodegenerative disorders. *Soc. Neurosci.* **23**, 738.3, abstract.

67. Hemmer, W., Zanolla, E., Furter-Graves, E., Eppenberger, H. M. and Wallimann, T. (1994): Creatine kinase isoenzymes in chicken cerebellum: specific localization of brain-type CK in Bergmann glial cells and muscle-type CK in Purkinje neurons. *Eur. J Neurosci.* **6**, 538–549.

68. Hemmer, W. and Wallimann, T. (1993): Functional aspects of creatine kinase in brain. *Dev. Neurosci.* **15**, 249–260.

69. Hemmer, W. and Wallimann, T. (1994): Creatine kinase in non-muscle tissues and cells. *Mol. Cell. Biochem.* **133/134**, 193–220.

70. Holtzman, D., Tsuji, M., Wallimann, T. and Hemmer, W. (1993): Functional maturation of creatine kinase in rat brain. *Dev. Neurosci.* **15**, 261–270.

71. Miller, K., Halow, J. and Koretsky, A. P. (1993): Phosphocreatine protects transgenic mouse liver expressing creatine kinase from hypoxia and ischemia. *Am. J. Physiol.* **265**, C1544–C1551.

72. Hatano, E., Tanaka, A., Iwata, S., Satoh, S., Kitai, T., Tsunekawa, S., Inomoto, B. and Yamaoka, Y. (1996): Induction of endotoxin tolerance in transgenic mouse liver expressing creatine kinase. *Hepatology* **24**, 663–639.

73. Brosnan, J. M., Chen, L., Wheeler, C. E., vanDyke, T. and Koretsky, A. P. (1991): Phosphocreatine protects ATP from a fructose load in transgenic mouse liver expressing creatine kinase. *Am J. Physiol.* C1191–C1200.

74. Pulido, S. M., Passaquin, A. C., Leijendekker W. J., Wallimann, T. and Rüegg, U. T. (1998): Creatine supplementation improves intracellular calcium handling and survival in mdx skeletal muscle cell. *FEBS Lett.* **439**, 357–362.

75. Brönnimann, M. and Wallimann, T. (1997): Creatine: break-through for the treatment of neuromuscular disorders? Swiss Soc. for Muscle Diseases. *Mitteilungsblatt* **43**, 3–10.

76. Stöckler, S., Hanefeld, F. and Frahm, J. (1996): Creatine replacement therapy in guanidinoacetate methyltransferase deficiency, a novel inborn error of metabolism. *Lancet* **348**, 789–790.

77. Martin, K. J., Winslow, E. R., O'Keefe, M., Khandekar, V. S., Hamlin, A., Lillie, J. W. and Kaddurah-Daouk, R. (1996): Specific targeting of tumor cells by the creatine analog cyclocreatine. *Internatl. J. Oncol.* **9**, 993–999.

78. Shoubridge, E. A., Bland, J. L. and Radda, G. K. (1984): Regulation of creatine kinase during steady-state isometric twitch contraction in rat skeletal muscle. *Biochim. Biophys. Acta* **805**, 72–78.

79. Goudemant, J. F., Francaux, M., Mottet, I., Demeure, R., Sibomana, M. and Sturbois, X. (1997): ^{31}P-NMR saturation transfer study of the creatine kinase reaction in human skeletal muscle at rest and during exercise. *Magn. Res. in Medicine* **37**, 744–753.

80. Hornemann, T., Stolz, M. and Wallimann, T. (1999): Interaction of muscle-type creatine kinase (MM-Ck) isoform with the myofibrillar M-band is mediated by four lysine residues located at the N-terminus. *J. Muscle Res. Cell Motil.* **20**, 112.

81. Soboll, S., Brdiczka, D., Jahnke, D., Schulze, K., Schmidt, A., Schlattner, U., Wendt, S. and Wallimann, T. (1999): Octamer-dimer transitions of mitochondrial creatine kinase in heart-disease. *J. Mol. Cell Cardiol.* **31**, 857-866.