Molecular and Cellular Biochemistry **133/134**: 51–66, 1994.

I-4 Creatine metabolism and the consequences of creatine depletion in muscle

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Abstract

Currently, considerable research activities are focussing on biochemical, physiological and pathological aspects of the creatine kinase (CK) – phosphorylcreatine (PCr) – creatine (Cr) system (for reviews see [1, 2]), but only little effort is directed towards a thorough investigation of Cr metabolism as a whole. However, a detailed knowledge of Cr metabolism is essential for a deeper understanding of bioenergetics in general and, for example, of the effects of muscular dystrophies, atrophies, CK deficiencies (e.g. in transgenic animals) or Cr analogues on the energy metabolism of the tissues involved. Therefore, the present article provides a short overview on the reactions and enzymes involved in Cr biosynthesis and degradation, on the organization and regulation of Cr metabolism within the body, as well as on the metabolic consequences of 3-guanidinopropionate (GPA) feeding which is known to induce a Cr deficiency in muscle. In addition, the phenotype of muscles depleted of Cr and PCr by GPA feeding is put into context with recent investigations on the muscle phenotype of 'gene knockout' mice deficient in the cytosolic muscle-type M-CK. (Mol Cell Biochem **133/134:** 51–66, 1994).

Key words: creatine kinase, mitochondrial myopathies, ragged red fibers, intramitochondrial inclusions, creatine analogues, phosphagen

Abbreviations: Cr – creatine; Crn – creatinine; PCr – phosphorylcreatine; CK – creatine kinase; M-CK – cytosolic muscle type CK isoenzyme; Mi-CK – mitochondrial CK isoenzyme; AGAT – L-arginine: glycine amidinotransferase; GAMT – S-adenosylmethionine: guanidinoacetate methyltransferase; Arg – arginine; Met – methionine; GPA – 3-guanidinopropionate = β-guanidinopropionate; PGPA – phosphorylated GPA; GBA – 3-guanidinobutyrate = β-guanidinobutyrate; CPEO – chronic progressive external ophthalmoplegia

Creatine metabolism

Although a diagram of the reactions involved in Cr metabolism (Fig. 1) seems simple, Cr metabolism is complicated by the fact that most tissues lack one or several of the reactions shown, necessitating a transport of intermediates between the tissues (through the blood) in order to allow the whole cascade to proceed. L-Arginine: glycine amidinotransferase (AGAT), the first enzyme in the two-step biosynthesis of Cr, catalyzes the transfer of the amidino group of Arg to glycine to yield L-ornithine and guanidinoacetate. The latter compound, by the ac-

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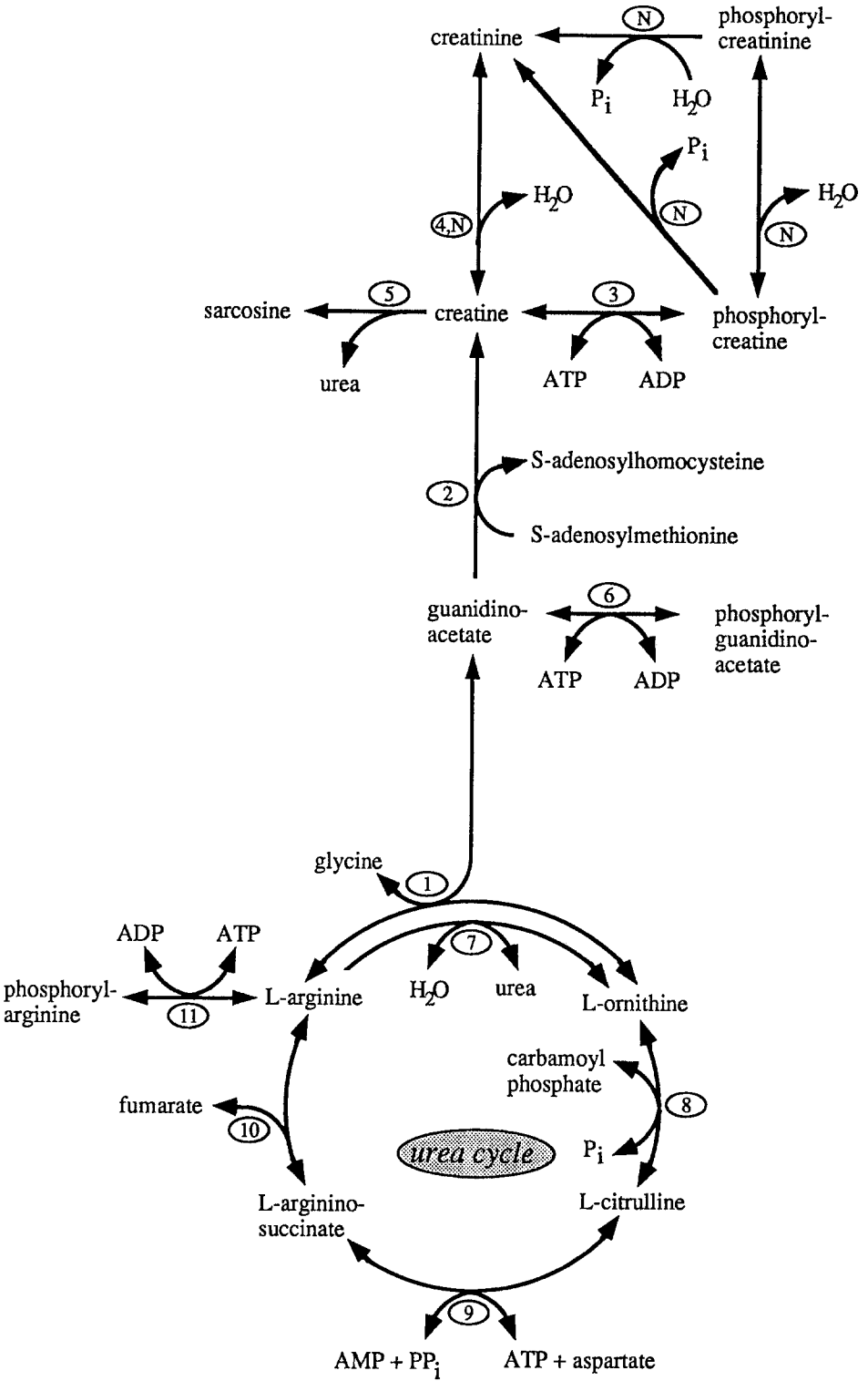

Fig. 1. Schematical representation of the reactions and enzymes involved in Cr metabolism. The respective enzymes are denoted by numbers: (1) L-arginine: glycine amidinotransferase (AGAT; EC 2.1.4.1); (2) S-adenosylmethionine: guanidinoacetate N-methyltransferase (GAMT; EC 2.1.1.2); (3) creatine kinase (CK, EC 2.7.3.2); (4) creatininase = creatinine amidohydrolase (EC 3.5.2.10); (5) creatinase = creatine amidinohydrolase (EC 3.5.3.3); (6) guanidinoacetate kinase = glycocyamine kinase (EC 2.7.3.1); (7) arginase (EC 3.5.3.1); (8) ornithine carbamoyltransferase (EC 2.1.3.3); (9) argininosuccinate synthetase (EC 6.3.4.5); (10) argininosuccinate lyase (EC 4.3.2.1); (11) arginine kinase (EC 2.7.3.3); (N) nonenzymatic reaction.

tion of S-adenosylmethionine: guanidinoacetate methyltransferase (GAMT), is then methylated at the amidino group to give Cr [3, 4]. In the course of evolution, both AGAT and GAMT seem to have evolved with the appearance of the lampreys [5]. While these enzyme activities were not detected in invertebrates, they were found in most, but not all vertebrates examined. Nevertheless, some invertebrate species (e.g. some annelids, echinoderms, hemichordates and urochordates, etc.) contain significant amounts of Cr, PCr and CK in their tissues, especially in spermatozoa [5–9], indicating that they either accumulate Cr from their environment or from the diet, or that the enzymes for Cr biosynthesis in these animals escaped detection so far.

Many of the lower vertebrates (fish, frogs and birds) express both AGAT and GAMT in their livers and often kidneys. In mammals, pancreas contains high levels of both enzymes, while kidneys have fairly high amounts of AGAT, but relatively lower levels of GAMT. On the contrary, all mammalian livers tested contain high levels of GAMT, but display only low levels of Cr, lack CK activity and consequently also PCr. While livers of cow, pig, monkey and man also have high amounts of AGAT, livers of common laboratory mammals such as the rat, mouse, dog, cat and rabbit were reported to lack AGAT activity (for reviews see [5, 10]). Based mostly on these latter findings and on the fact that nephrectomized animals display a drastically reduced rate of Cr synthesis [11, 12], it was postulated and is still largely accepted that the main route of Cr biosynthesis in mammals involves the formation of guanidinoacetate in the kidney, its transport through the blood and its methylation to Cr in the liver. Cr exported from the liver and transported again through the blood may then be taken up by the Cr-requiring tissues.

There is, however, experimental evidence not fully agreeing with this view. Immunofluorescence microscopy with antibodies against AGAT revealed significant amounts of this enzyme not only in rat kidney and pancreas, but also in liver [13]. The underestimation of rat liver AGAT in previous biochemical studies was most likely due to the high levels of liver arginase interfering with the AGAT assay. Furthermore, AGAT activity was detected in heart, lung, spleen, muscle, brain, testis and thymus, and the total amount of AGAT in these tissues was estimated to even approach the AGAT activity in kidney and pancreas [14]. In the rat, the highest specific AGAT activity was found in the decidua of pregnant females [10], but AGAT is absent from human placenta. GAMT activity, on the other hand, was also detected in rat spleen, heart and skeletal muscle as well as in human fetal lung fibroblasts and mouse neuroblastoma cells [15, 16], but the specific activities in these tissues are rather low. The GAMT activity in skeletal muscle, however, was calculated to have the potential to synthesize all Cr needed in this tissue [16]. Finally, feeding of rats and mice with GPA, a competitive inhibitor of Cr entry into cells, progressively decreases the concentrations of Cr and PCr in heart and skeletal muscle, but has only little influence on the Cr and PCr contents of brain [17; Riesinger, Haas & Wallimann, unpublished results]. One possible explanation is that brain contains its own Cr-synthesizing machinery [18]. To conclude, the detailed contribution of various tissues to total Cr synthesis on one hand as well as the relevance of guanidinoacetate and Cr transport through the blood and of Cr uptake by the cells on the other hand are still not fully understood, this being due to a lack of thorough investigations and to pronounced species differences.

A specific saturable, Na^+- and Cl^--dependent Cr transporter has been described for skeletal muscle, heart, fibroblasts, smooth muscle, neuroblastoma and astroglia cells, as well as for red blood cells and macrophages [16, 19–27]. Recent cloning of the Cr transporter cDNA, followed by Northern blot analysis, revealed the largest amounts of Cr transporter mRNA in kidney, heart and skeletal muscle, somewhat lower levels in brain, lung, epididymis and testis, but no Cr transporter mRNA at all in uterus, liver, small intestine and spleen [27].

As far as the tissue concentrations of Cr and PCr are concerned, the highest levels were observed in skeletal muscle, heart, spermatozoa and photoreceptor cells of the retina, somewhat lower amounts in brain, brown adipose tissue, intestine, seminal vesicles and seminal vesicle fluid, and only low levels in lung, spleen, kidney, liver, white adipose tissue, erythrocytes and serum [15, 28–36]. In skeletal muscles, [Cr] and [PCr] correlate with the glycolytic capacity, with resting type 2a and 2b fibers containing approx. 32 mM PCr and 7 mM Cr, and type 1 fibers containing approx. 16 mM PCr and 7 mM Cr [36, 37]. In serum and erythrocytes, on the other hand, [Cr] amounts to only 25–50 μM and 270–400 μM, respectively [35], implying that Cr has to be accumulated by most Cr-containing tissues against a large concentration gradient from the blood. Very likely, Cr uptake via the Cr transporter is driven by the electrochemical potential difference of extracellular versus intracellular $[Na^+]$.

As indicated in the upper part of Fig. 1, several path-

ways have to be considered for the biodegradation of Cr and PCr. *In vitro,* reversible and nonenzymatic cyclization of Cr to creatinine (Cr ↔ Crn) takes place, with the equilibrium of this spontaneous reaction being both pH- and temperature-dependent. Cr is favoured at low pH and low temperature, while Crn is favoured at higher temperatures and in alkaline solutions [38, 39]. In both directions, the reaction is monomolecular. Starting with pure Cr solutions, 1.0–1.3% of the Cr per day converts to Crn at pH 7.0–7.2 and 38 °C. *In vitro* studies on the stability of PCr revealed that this 'high-energy phosphate compound' is acid-labile, yielding P_i and either Cr or Crn upon hydrolysis. Both the rate of PCr hydrolysis and the ratio of Cr to Crn formed depend on temperature and pH and can additionally be influenced in a concentration-dependent manner by molybdate (for reviews see [7, 40]).

In contrast to the *in vitro* situation, studies with ^{15}N-labelled Cr and Crn clearly demonstrated that the conversion of Cr to Crn *in vivo* is an *irreversible* process [41]. An almost constant fraction of the body Cr (1.1%/day) and PCr (2.6%/day) nonenzymatically converts to Crn *in vivo,* giving an overall conversion rate for the total Cr pool (Cr + PCr) of approx. 1.7%/day [42; for a review see ref. 10]. Consequently, in a 70 kg-man containing approx. 120 g of total Cr, roughly 2g/day are converted into Crn and have to be replaced by Cr from the diet or by de-novo biosynthesis [10, 42, 43]. Since in contrast to Cr, no specific saturable uptake mechanism exists for Crn [24], and since Crn, most likely due to its nonionic nature, is membrane-permeable, Crn constantly diffuses out of the tissues into the blood and is excreted by the kidneys into the urine [29].

20–25% of the *in vivo* conversion of PCr into Crn may proceed via phosphorylcreatinine (PCrn) as an intermediate [44]. Accordingly, [PCrn] in rabbit white skeletal muscle was found to be 0.4% of [PCr]. In addition, commercial preparations of PCr contain 0.3–0.7% of PCrn. Although PCrn was proposed to be an obligatory intermediate of the CK reaction [45], this idea has to be dismissed due to the lack of any experimental evidence.

In contrast to the nonenzymatic conversion of Cr and PCr to Crn in vertebrates, various bacteria (Alcaligenes, Arthrobacter, Clostridium, Flavobacterium, Micrococcus, and Pseudomonas strains) were shown to (inducibly) express specific enzymes for the biodegradation of Cr and Crn ([46–51]; for further references see [52]). In some of these bacteria, for example, creatininase (Crn amidohydrolase) first converts Crn into Cr which then is further metabolized by creatinase (Cr amidinohydrolase) into urea and sarcosine. Even though creatinase was also detected in human skeletal muscle [53], this finding awaits confirmation and demonstration of its physiological relevance. Very interesting, however, is the indication that in Duchenne muscular dystrophy, the kinetic properties of human muscle creatinase are affected [54].

An important aspect of Cr metabolism to add is that in man, the daily utilization of methyl groups for Cr synthesis (in the GAMT reaction) and, consequently, also the daily loss of methyl groups due to Crn (and Cr) excretion approximately equal the daily intake of 'labile' methyl groups (Met + choline) on a normal, equilibrated diet containing Met and choline [55]. Even when de novo Met biosynthesis is taken into consideration, Cr biosynthesis still accounts for approx. 70% of the total utilization of 'labile' methyl groups. It might therefore be assumed that methyl group availability becomes limiting for Cr biosynthesis, at least under some physiological or pathological conditions. This is, actually, not the case, since a deficit in 'labile' methyl groups in man will normally be compensated by increased de novo Met biosynthesis.

Regulation of creatine metabolism

Despite the relatively simple scheme of Cr metabolism (Fig. 1), a variety of potential regulatory mechanisms have to be considered (for an extensive review see [10]), for instance allosteric regulation, covalent modification or alterations of expression of enzymes involved in Cr metabolism, or changes in the diffusion and transport properties (membrane barriers, transport proteins, blood transport) of intermediary metabolites.

Formation of guanidinoacetate is rate-limiting for Cr biosynthesis (see [10]). Consequently, the AGAT reaction is the most likely control step in the pathway, a hypothesis that has been proven by a great deal of experimental work. Most important in this respect is the feedback repression of AGAT by Cr, the endproduct of the pathway. An increase in endogenous or exogenously supplied Cr causes a parallel decrease in the mRNA content, the enzyme level, as well as the specific activity of AGAT, suggesting regulation of AGAT expression at a pretranslational level ([56, 57]; for a review see [10]). Feedback repression of AGAT by Cr is most pronounced in kidney and pancreas, the main tissues of guanidinoacetate formation, but is also observed in the decidua of the pregnant rat (see [10]). Cyclocreatine, N-

acetimidoylsarcosine and N-ethylguanidinoacetate also display repressor activity like Cr, while Crn, PCr, N-methyl-3-guanidinopropionate and a variety of other compounds are ineffective [10, 58, 59]. L-Arg and guanidinoacetate have only 'apparent' repressor activity, since they have no effect on AGAT expression by themselves, but are readily converted into Cr which then acts as the true repressor. Since the half-life of AGAT in rat kidney is two to three days [56], the changes in the AGAT levels described here are rather slow processes, thus only allowing for long-term adaptations. Furthermore, immunological studies suggested the presence of multiple forms (isoenzymes?) of AGAT in rat kidney, of which only some are repressible by Cr [60].

The expression of AGAT was suggested to be modulated not only by Cr, but also by dietary and hormonal factors (for reviews see [10, 61]). Dietary deficiencies (fasting, protein-free diets) and diseases (vitamin E deficiency, spreptozotocin-induced diabetes; [62, 63]) decrease AGAT levels in liver, pancreas and kidney. During fasting and in vitamin E deficiency, however, at least part of the observed effect may be explained by the increased blood levels of Cr ([12]; see also [10]). Furthermore, kidney AGAT activity is reduced upon thyroidectomy or hypophysectomy of rats [64]. The original AGAT activities can be restored by injection of thyroxine or growth hormone, respectively. In contrast, injections of growth hormone into thyroidectomized rats and of thyroxine into hypophysectomized rats are without effect, implying that both hormones are necessary for maintaining proper levels of AGAT in rat kidney. Since enzyme activity, protein and mRNA contents are always affected to the same extent, regulation of AGAT expression by thyroid hormones and growth hormone also occurs at a pretranslational level, very similar to the feedback repression by Cr [57, 65]. Growth hormone and Cr have an antagonistic action on AGAT expression, as evidenced by identical mRNA levels and enzymatic activities of kidney AGAT in rats fed Cr and injected with growth hormone as compared to rats receiving neither of these compounds [57]. Finally, AGAT levels in rat testes and decidua are presumably under the control of sex hormones, with estradiol and diethylstilbesterol decreasing and testosterone increasing the AGAT levels (see [10]).

In contrast to the repression of AGAT, Cr does not interfere with the expression of GAMT or arginase in liver. Cr, Crn and PCr also do not act as allosteric regulators of the enzymatic activities of AGAT or GAMT *in vitro* [10], suggesting that feedback regulation is achieved exclusively by the action of Cr on the rate of AGAT biosynthesis. AGAT is, however, very efficiently inhibited by ornithine, a fact that seems to be pathologically relevant [10, 66]. In gyrate atrophy of the choroid and retina, for instance, plasma ornithine concentrations are increased 10-20-fold due to deficiency of L-ornithine: 2-oxo-acid aminotransferase [67]. The increased ornithine concentrations, in turn, inhibit AGAT and thus decrease the rate of guanidinoacetate formation from Arg, resulting in drastically decreased serum concentrations of Cr and Crn [68].

As far as regulation of transport processes involving intermediates of Cr metabolism is concerned, many potential points of attack have to be considered, for instance uptake of Arg into mitochondria, release of guanidinoacetate from pancreas and kidney, uptake of guanidinoacetate into and release of Cr from the liver, uptake of Cr into the tissues, and penetration of ATP, ADP and PCr through the mitochondrial membranes. In chicken kidney and liver where AGAT is localized in the mitochondrial matrix, penetration of L-Arg through the inner membrane was found to occur only in respiring mitochondria and only in the presence of anions such as acetate or phosphate [69]. Consequently, the rate of Cr synthesis in the chicken may be influenced by the rate of penetration of Arg into the matrix space. The uptake of blood Cr by muscle was shown to be stimulated by insulin (see [10]). In contrast, the Cr transporter activity in rat and human myoblasts and myotubes is down-regulated by extracellular Cr [70].

The permeability itself as well as changes in permeability of the outer mitochondrial membrane may be critical for the stimulation of mitochondrial 'high-energy phosphate' synthesis and for the transport of these 'high-energy phosphates' between sites of ATP production and ATP utilization within the cell [71, 72]. Changes in the permeability of the outer mitochondrial membrane pore protein (VDAC) may be achieved by 'capacitive coupling' to the membrane potential of the inner mitochondrial membrane, leading to a voltage-dependent 'closure' of the pore (for a review see [73]), or by the recently discovered VDAC modulator protein which increases the rate of voltage-dependent channel closure by approximately 10-fold [74]. Since upon stimulation of mitochondrial respiration from state 4 to state 3, the number of contact sites between mitochondrial inner and outer membranes increases (see [73]), capacitive coupling between the two membranes may be favoured, and the pore protein may be shifted from its open, anion-selective to its closed, cation-selective state.

Finally, increased Cr levels in the blood cause increased guanidinoacetate excretion in the urine, presumably by inhibiting reabsorption of guanidinoacetate by kidney tubules (see [10]). To conclude, the theoretical possibilities of regulating Cr metabolism are manifold. Practically, the highest rates of Cr biosynthesis are observed in young, healthy, fast-growing vertebrates in optimal hormonal balance and under anabolic conditions with a high-quality, Cr-free food supply [10]. The best established regulatory mechanism in Cr metabolism so far is feedback repression of AGAT by Cr. An attractive further possibility, which is testible more easily now after the recent identification and cloning of the Cr transporter, represents a reversible up- and down-regulation of this transporter as a function of dietary Cr intake.

Interference with creatine uptake into muscle

Muscle phenotype after chronic creatine depletion

Animal models for cardiac hypertrophy [75, 76] and diseased human myocardium [77] are characterized by lowered overall intracellular [Cr] and [PCr] as well as by a higher vulnerability to hypoxia. Consequently, lowering of the energy reserves for ATP synthesis may render muscle more susceptible to failure. The same holds true also for a number of skeletal muscle myopathies [78] in which a disturbance in transport and handling of Cr is indicated.

Therefore, feeding of experimental animals with Cr analogues would seem a promising tool to test whether lowered levels of total Cr are related to pathological muscle function, and to investigate the physiological role of PCr, Cr and CK in intact muscle. In order to provide clear-cut answers, an 'ideal' Cr analogue should i) either completely inhibit Cr biosynthesis, ii) completely prevent Cr uptake by muscle and nerve *in vivo,* iii) completely and specifically inhibit CK activity *in vivo,* or iv) completely replace Cr and PCr, with the phosphorylated synthetic analogue possessing markedly different thermodynamic and kinetic properties relative to PCr [10]. Unfortunately, none of the Cr analogues studied so far fulfils any of these criteria.

The frequently used Cr analogues 3-guanidinopropionic acid (β-guanidinopropionic acid; GPA) and 3-guanidinobutyric acid (β-guanidinobutyric acid; GBA) both competitively inhibit the Cr transporter activity and thus reduce Cr import through the sarcolemma, with GBA being somewhat less effective than GPA [10, 79–85]. Compared to GPA, however, GBA has the advantage not to be phosphorylated by CK *in vivo* [85]. Long-term feeding (6–10 weeks) of rats with GPA results in a marked decrease in PCr, Cr and ATP levels in skeletal muscle to approximately 10, 20 and 50% of normal, respectively [83, 86]. At the same time, GPA and its phosphorylated counterpart, PGPA, are accumulated at high concentrations, especially in white, fast-twitch skeletal muscles. In spite of the severely reduced PCr levels, these muscles continue to function reasonably well [79, 81], i.e. neither the initial peak tension nor the long-term steady-state force developed at low workloads are significantly reduced [83]. This, however, does not surprise the informed reader: i) Although the levels of PCr are decreased drastically, the calculated unidirectional flux from PCr to ATP via the CK reaction in resting analogue-loaded muscle (0.5 $\mu mole \cdot g^{-1} \cdot s^{-1}$) is still severalfold greater than the steady-state ATP turnover rate at rest (0.07 $\mu mole \cdot g^{-1} \cdot s^{-1}$) [83]. And ii), the rate of PGPA break-down by CK (0.18 $mM \cdot s^{-1}$) is still in excess of the ATPase rate during a transition from low to high work load (0.1 $mM \cdot s^{-1}$) [87]. This is due to GPA and PGPA both serving as substrates for CK to some extent [88]. The V_{max} values of CK for GPA and PGPA are approximately 0.3% and 0.01% of those for Cr and PCr, respectively [89].

Considering these facts, interpretations of results obtained with GPA-treated animals that have been put forward, like 'PCr is not essential for steady-state energy production' [83] or 'neither PCr nor the activity of CK is critical for aerobic metabolism' [81], must be considered with well founded dubiety. As a matter of fact, a closer look at Cr-depleted muscle reveals considerable deviations in contractile properties. In Cr-depleted rat diaphragm muscle during a burst of intense muscle activity (0.2-s tetanic stimulation every 0.5 s), the maximum isometric tension, rate of tension development and rate of relaxation decrease rapidly to reach a minimum about 3 s after the onset of activation [90, 91]. In contrast, normal muscles show a small decrease in tension and relaxation rate but an increase in the rate of tension development under these conditions. Similar findings, although interpreted differently, were made with rat skeletal muscle [83]. During 3-Hz stimulation, hypoxic tibialis anterior muscle of GPA-treated rats is characterized by a rapid decline in peak tension and by the absence of the so-called staircase phenomenon [88]. Post-tetanic stimulation, a common phenomenon seen in normal muscle after a 1-s tetanus, is largely reduced in GPA-loaded

EDL muscle [92]. Furthermore, GPA-loaded soleus muscle displays altered isometric twitch characteristics, in particular a decrease in the maximum velocity of shortening as well as a prolonged half-relaxation time [93]. A major effect of Cr depletion on excitation-contraction coupling, specifically on the relaxation rate, is observed in hearts of GPA- and GBA-treated animals, especially at higher work loads [94, 95]. Long-term feeding of rats with GPA and GBA also causes a decrease in the cytosolic phosphorylation potential [83, 85] and a decrease in the thermodynamic efficiency of cardiac energy metabolism [85]. Finally, the effects of Cr depletion can be markedly exacerbated by superimposing a thyrotoxicosis, e.g. by simultaneously feeding GPA and thyroid powder. Much more severe muscle degeneration is observed under these conditions than with GPA feeding or thyroid powder supplementation alone, indicating that high concentrations of Cr and PCr are essential for the maintenance of muscle integrity during periods of metabolic stress [96].

Many of the findings listed above indicate that muscle relaxation is affected in analogue-treated animals. This is most likely due to an impairment of proper Ca^{2+} handling, since i) muscle relaxation afforded by Ca^{2+}-sequestration into the sarcoplasmic reticulum (SR) depends critically on a highly negative ΔG for ATP hydrolysis in the cytosol [97, 98], and since ii) the ΔG for ATP hydrolysis is less negative in analogue-loaded than in control muscle, due mainly to an increase in [free ADP] [83, 85]. Fully in line with this interpretation, a fraction of the cytosolic CK is bound to the SR and is functionally coupled to the SR-Ca^{2+}-ATPase [99, 100]. In this location, CK was proposed to locally regenerate ATP and thus to maintain a high phosphorylation potential in the intimate vicinity of the Ca^{2+} pump of the SR [1].

Metabolic adaptation of muscle chronically depleted of creatine

The altered contractile properties of muscle, chronically depleted of Cr, are reflecting the effects of the defect itself (Cr, PCr and thus 'high-energy phosphate' deficiency) plus a superimposition of effects caused by compensatory, qualitative and quantitative adaptational changes induced by the Cr-depletion. The astonishing plasticity of muscle to adapt to specific requirements under physiological conditions is well documented and can be nicely demonstrated by chronic stimulation of fast glycolytic muscle fibers which are readily converted into slow oxidative fibers [101, 102]. Obviously, this muscle plasticity can also be provoked by artificial stimuli. In the case of Cr-depletion due to GPA or GBA feeding, fast-twitch skeletal muscle fibers adapt to a 'high-energy phosphate' deficit i) by reducing fiber diameter, resulting in reduced diffusion distances for 'energy' metabolites [103], ii) by increasing aerobic capacity [104, 105], iii) by decreasing their glycolytic potential, with glycogen content increasing at the same time, iv) by increasing the proportion of slow-twitch fibers in skeletal muscle [103], and v) by shifting the myosin isoform pattern from fast to slow isomyosins [106]. The finding of a similar shift in cardiac muscle from the faster ventricular myosin V_1 to the slower V_2 isoform [84] contrasts with an earlier study where no changes in ventricular isoforms were reported [106].

Consistent with the notion that the oxidative capacity of Cr analogue-treated muscle is increased, long-term GPA feeding causes a 60–67% increase in cytochrome c mRNA in rat soleus and white quadriceps muscle [104] as well as a 40–50% increase in cytochrome c, citrate synthase and hexokinase activity in rat plantaris muscle [105]. Furthermore, a 50% increase in the major glucose transporter isoform in skeletal muscle, GLUT-4, was seen in Cr-depleted muscle [105]. Since GLUT-4 is the major determinant of a muscle's maximal insulin-stimulated glucose transport capacity, all these findings point to a metabolic adaptation to increase the availability of energy sources (ATP) for proper cell function. Finally, AMP deaminase activity is significantly reduced, specifically in fast-twitch muscle fibers, to a level normally found in slow-twitch muscles [107].

To conclude, chronic depletion of Cr in muscle clearly results in a multiplicity of stratified metabolic adaptations. However, the very interesting questions of how and by which signalling cascades a low-energy stress situation is transmitted to bring about the induction of compensatory measures still await an answer. On the other hand, much care should be taken in the interpretation of results obtained with animal models of long-term Cr depletion.

In order to circumvent the problem of metabolic and structural adaptations, an acute *ex vivo* model has recently been established using perfusion of isolated hearts with 150 mM GPA [108]. In this short-term model, a linear accumulation of PGPA is accompanied by a 30% decrease of PCr over a 2 hr period. The increase in P_i and the decrease in ATP which occur concomitantly with PGPA accumulation indicate that ATP synthesis is not keeping up with ATP demand. Short-term GPA per-

fusion reduces the cardiac frequency and developed tension by approx. 40% and 10%, respectively. Perfusion with 150 mM mannitol instead of GPA results in a 15% decrease in cardiac frequency, with a similar decrease in ATP, increase in intracellular pH and a smaller rise in P_i being observed with mannitol compared to GPA. These results suggest that some of the effects observed with the acute GPA model are due to the hyperosmolarity caused by 150 mM GPA. Therefore, the scientific merit of this method of Cr depletion has still to be evaluated thoroughly.

Changes of mitochondrial structure seen in creatine-depleted muscle

As *in vivo,* muscle cells in culture seem to depend on Cr for proper differentiation and cell function [109]. Cultivation of adult rat cardiomyocytes *in vitro* in a Cr-deficient medium or in the presence of GPA results in marked morphological changes, mainly affecting mitochondria [110]. After 3–4 days in culture, a population of enlarged, rod-shaped mitochondria with characteristic crystalline intramitochondrial inclusions appears in these cells. This phenomenon is fully reversible if the cell culture medium is supplemented with Cr. The appearance of highly ordered intramitochondrial inclusions correlates with a low intracellular total Cr content of the cardiomyocytes [110]. Most importantly, the large, rod-shaped mitochondria react very strongly with specific anti-mitochondrial creatine kinase (Mi-CK) antibodies in immunofluorescence experiments. Higher-magnification immuno-electron microscopy showed that, in fact, the highly ordered intramitochondrial inclusions are heavily enriched for Mi-CK [110].

Very similar intramitochondrial inclusions have been observed in several animal models: in skeletal muscle of rats fed with a diet containing 1–2% GPA [111–113]; in adult ventricular cardiomyocytes, cultured for six days in serum-supplemented medium, followed by serum-free medium containing the α_1-adrenoceptor agonist, phenylephrine (stimulating protein synthesis) [114]; in ischemic rat skeletal muscle *in vivo* [115]; in organ cultures of rat diaphragm, most likely suffering from anoxic conditions [116]; as well as after acute *in vivo* administration of uncouplers of oxidative phosphorylation [117]. It is tempting to speculate that all of these intramitochondrial inclusions contain Mi-CK as main or even sole component [110].

GPA administration to rats for 6–10 weeks induces two types of intramitochondrial inclusions, mainly seen in enlarged subsarcolemmal mitochondria of red skeletal muscle and diaphragm [111–113, 118]; long ribbon-like peripheral inclusions (Fig. 2A), and regular staples of intracristae inclusions often arranged in packages consisting of two or four distinct tracks (Fig. 2B). Each track contains periodically arranged 'material' that is fitted between or connects two adjacent cristae membrane folds (Fig. 2B). The ensheathment of the periodic material by the cristae membrane can most clearly be seen at the ends of individual tracks (Fig. 2B, arrows). Often, if the tracks are very long, they tend to circularize or break (Fig. 2B, arrowhead). Detailed inspection of high-magnification electron micrographs suggests that the inclusions are generated by the close apposition of two folds of the mitochondrial inner membrane, with the crystalline material accumulating in between, first as one single layer [119] and then building up to more compact structures [111–113, 115, 118] (See also Fig. 2). The findings that i) the highly symmetrical octameric Mi-CK molecules are able to link two membranes and to stabilize such membrane contacts *in vitro* [120] and that ii) octameric Mi-CK under certain experimental conditions 'polymerizes' to form ribbon-like linear filaments [121] suggest that the intramitochondrial inclusions seen in GPA-treated experimental animals, like those observed in Cr-depleted cardiomyocytes in culture, also consist mainly of Mi-CK. This hypothesis has recently been corroborated by preliminary immunogold labelling experiments [118, Gorman et al., unpublished].

Mitochondrial creatine kinase is a major constituent of pathological inclusions seen in biopsies of human patients with mitochondrial myopathies

Most interestingly, mitochondrial inclusions are frequently seen in patients with so-called mitochondrial encephalomyopathies. These mitochondrial myopathy diseases [122] are characterized by the presence of mutations in the mitochondrial DNA which affect in some way or another ATP production by oxidative phosphorylation [123–125]. These defects are particularly revealed in tissues such as skeletal muscle, heart and brain which rely the most on oxidative phosphorylation. The structural hall-mark of these syndromes is the presence of 'ragged red muscle fibers' in muscle biopsies [126, 127]. Characteristic aspects of 'ragged red muscle fibers' are an accumulation of enlarged and abnormal mitochondria and the occurrence in these mitochondria of

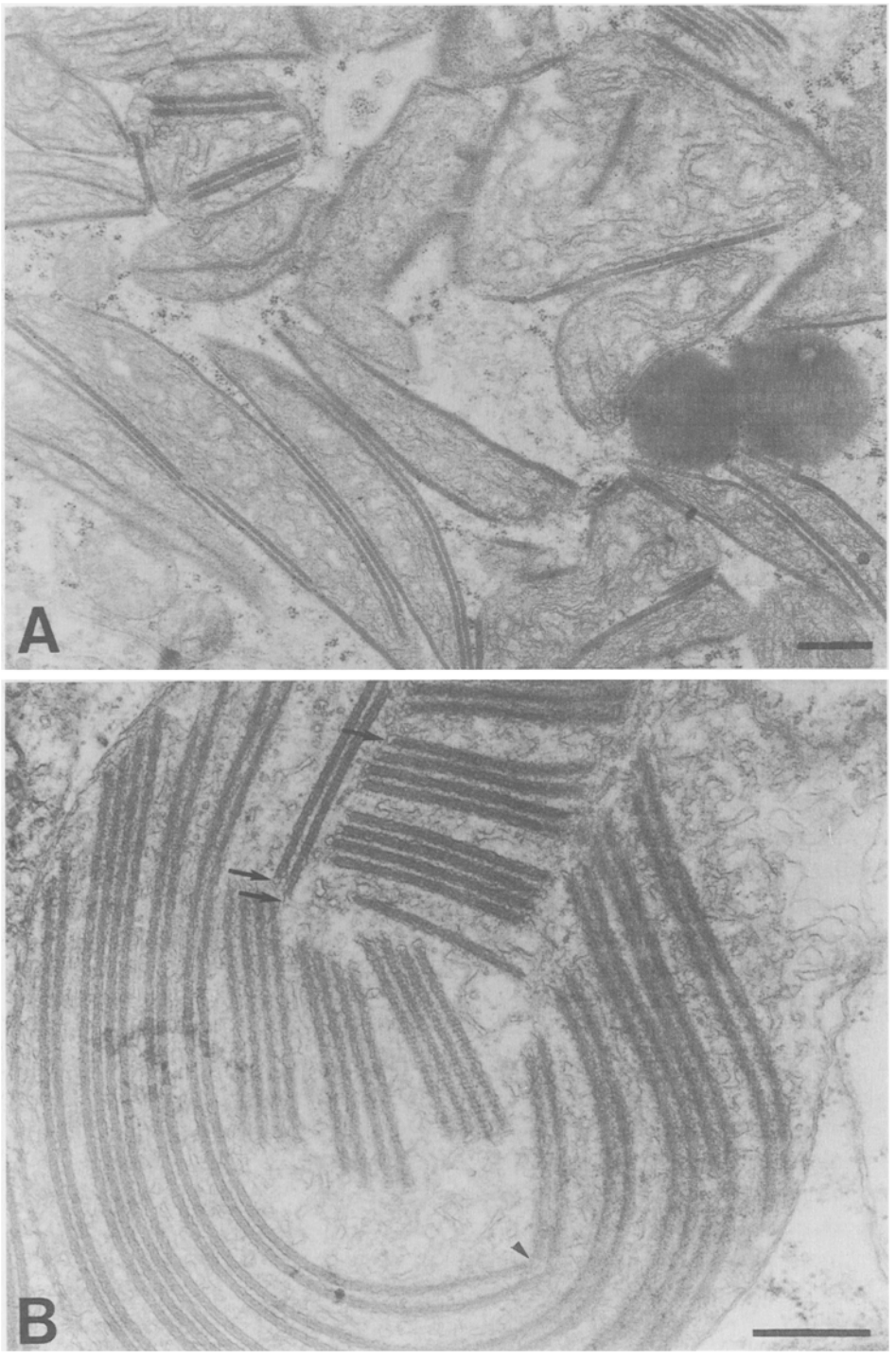

Fig. 2. GPA-induced morphological changes in muscle mitochondria. Intramitochondrial crystalline inclusions were induced in rat muscle by 10 weeks of supplementation of the normal rat food with 2% (w/w) of 3-guanidinopropionic acid (GPA), a creatine uptake inhibitor. A) Rat diaphragm muscle with enlarged mitochondria and inclusions located beneath the mitochondrial outer membrane (peripheral inclusions). B) Rat diaphragm muscle with enlarged roundish mitochondria and inclusions located between cristae membranes (intracristae inclusions). Note pairs of two or four 'ribbons' appearing either as short stacks or alternatively as long structures that are often curved or kinked (arrowhead). At the end of some of the ribbons, the cristae membrane enveloping the crystalline material can be identified (arrows). Bars = 0.4 μm (courtesy of C. Haas and Dr. I. Riesinger, see [118]).

Fig. 3. Intramitochondrial inclusions in biopsies of patients with mitochondrial myopathies consist mainly of mitochondrial creatine kinase. A section of a skeletal muscle biopsy (from m. quadriceps) from a patient with chronic progressive external ophthalmoplegia (CPEO) was stained with specific anti-mitochondrial creatine kinase (Mi-CK) antibodies, followed by gold-conjugated second antibody. Note the strong and specific immunostaining of the intramitochondrial inclusions even after standard EM embedding techniques. Bar = 0.1 μm (courtesy of Prof. Ad M. Stadhouders, see [132]).

highly ordered crystal-like inclusions [128–130]. These crystals are very reminiscent of those seen in the experimental GPA-animal model described above.

The structural features of the crystalline intramitochondrial inclusions, observed in biopsy material mostly from patients with chronic progressive external ophthalmoplegia (CPEO), have been studied in great detail by electron microscopy of oriented thin sections [131]. Analysis of the electron micrographs by image processing revealed regularly packed, square-shaped building blocks of 10 nm side width and a central channel [132], features matching the structural characteristics of isolated Mi-CK octamers [121]. Recent immunogold labelling experiments showed that the mostly proteinaceous in-

clusions in CPEO patients react very strongly with specific anti-Mi-CK antibodies [132, 133] (see Fig. 3).

Functional aspects of intramitochondrial Mi-CK-containing inclusions

An interesting aspect of a metabolic adaptation has recently been elucidated. In skeletal muscles of patients with defects in and low expression of mitochondrial genes involved in oxidative phosphorylation, nuclearly encoded mitochondrial proteins involved in ATP production are overexpressed to compensate for the respiratory deficiency [134]. Thus, one would like to propose that the Mi-CK-rich inclusions seen in patients with mitochondrial myopathies are generally formed as a response to a chronic cytosolic 'low energy' stress situation. It will be extremely interesting to find the sensors as well as signalling factors responsible for the induction of these compensatory mechanisms. Whether the Mi-CK inclusions in fact serve a compensatory ameliorating function, i.e. by increasing the export of 'high-energy phosphates' from mitochondria to the cytosol (increased V_{max}/K_m ratio), remains to be elucidated. However, this idea would be fully compatible with the proposed function of Mi-CK as an energy channelling molecule [2, 135]. The metabolic compensation hypothesis is also supported by enzyme histochemical methods, showing that Mi-CK is enzymatically active even within fully grown intramitochondrial crystals [132]. At an advanced stage, on the other hand, the crystallization of massive amounts of Mi-CK within cristae folds or between inner and outer mitochondrial membranes may have to be considered pathological itself, for it is difficult to envisage how such crystals could improve functional coupling of Mi-CK with the adenine nucleotide translocator of the inner and with porin of the outer membrane [2, 135, 136].

Transgenic null mutants for cytosolic M-CK reveal similar phenotype and metabolic adaptation as seen in creatine-depleted muscle

Very recently, a null mutation for the cytosolic muscle CK (M-CK) gene was created in transgenic mice [137]. These M-CK knockout mutant mice, despite the complete absence of M-CK mRNA as well as active M-CK enzyme, still express more or less normal levels of Mi-CK and show normal concentrations of free ATP, PCr and P_i in their resting muscles. Most strikingly, the well-known symmetrical changes of [PCr] and [P_i] (a decrease in one is paralleled by an increase in the other) during exercise and recovery are similar in the mutants and controls. Nevertheless, upon closer examination of muscle performance, a clear physiological phenotype becomes apparent. The mutant mice lack the ability to perform burst muscle activity, that is, although their muscles reach normal initial peak tension, they are unable to maintain it for any length of time. Transgenic mice lacking M-CK compensate for the deficiency by structural and metabolic adaptations in their muscles, e.g. by increasing mitochondrial capacity and glycogen content in fast muscle fibres. The expansion of the mitochondrial network in mutant fast-twitch fibres, e.g. in the subsarcolemmal and intermyofibrillar space, results in a considerable reduction of diffusion distances between mitochondria and myofibrils. These findings point to an adaptation towards increased energy transport mediated directly via ATP and ADP [137].

Thus, M-CK-deficient transgenic mice (with no cytosolic CK, but normal substrate concentrations; [137]) and GPA- or GBA-treated animals (with normal CK activity, but decreased substrate concentrations [82, 83, 90–92]) display a notably similar phenotype as far as physiological muscle performance is concerned. In both animal models, i) the initial peak muscle tension reached is normal, ii) the peak force, however, cannot be maintained and declines rapidly after the onset of muscle stimulation, i.e. muscle burst activity is affected, and iii), marked compensatory adaptive changes take place, leading to an improvement of endurance performance of the muscles [137]. This muscle phenotype is exactly what one would expect if the high-energy phosphate buffering function, facilitated by PCr and cytosolic CK, were hampered. Therefore, both animal models provide additional confirmation for the postulated role of cytosolic M-CK as an immediate 'high-energy phosphate' buffer for short-time muscle activity (see [1, 136]).

However, the enhancement in aerobic capacity of M-CK-deficient mice is afforded mainly by an increase in the number of relatively large intermyofibrillar mitochondria, and only rarely are intramitochondrial inclusions seen [137]. By contrast, in GPA-treated animals, grossly enlarged mitochondria accumulate mostly in a clustered fashion in the subsarcolemmal space and frequently display prominent intramitochondrial crystals [111–113, 115, 118] (see Fig. 3). Although in the M-CK 'knock-out' animals, the fast-twitch type 2 muscle fibers have an increased intra-myofibrillar mitochondrial vol-

ume and an increased glycogenolytic/glycolytic potential, and in spite of the adaptation of the muscles to endurance performance, no signs of muscle fiber-type conversion, as seen in GPA-treated animals, have been observed in M-CK-deficient mice [137].

Concluding remarks

The accumulation of the unique type of intramitochondrial inclusions described above is a common denominator seen in a variety of diseases linked to defects in mitochondrial energy metabolism as well as in animal models with a depleted energy status. Since these inclusions contain Mi-CK as their major component or may even be 'pure' Mi-CK protein crystals, their occurrence in pathology points to the physiological importance of the CK/PCr system for cellular energetics. These findings further impose the intriguing question of how a defect in cellular energy metabolism (substrate *or* enzyme deficiency) can regulate and influence muscle plasticity towards long-term structural and metabolic adaptation.

Seen in the broad context of system physiology, organ and cell function, the pathways and regulation of Cr biosynthesis and degradation are very fascinating, but unexpectedly complex, with many basic questions still open. Cr and CK, however, have managed to make a come-back and obtain at least a glimpse at the lime-light of modern biological science. The surprising findings made with M-CK null-mutant transgenic mice and the results to be expected in the near future with 'knock-out' mutants of the other CK isoenzymes, which most likely also will display distinct phenotypes, have already provided a deeper insight in CK function *in vivo* and are likely to shed new light on Cr, PCr and CK function in the intact animal, respectively. Above all, these types of experiments will keep the next generation of cell, organ and system physiologists (if there are any left?) quite busy in the future.

Acknowledgments

We would like to thank Prof. Ad M. Stadhouders, Institute for Cell Biology at the University of Nijmegen, Holland for providing figure 3 and to C. Haas and Dr. I. Riesinger, Institute for Cell Biology, ETH Zürich, for the negatives for figure 2. Thanks are also due to Eddie O'Gorman for carefully reading the manuscript and to Dr. E. Gnaiger, Innsbruck, Austria, for discussion. This work was supported by the Swiss National Science Foundation (SNF grant 31-33907.92 to T.W.), a postdoctoral SNF training grant (823A-037106 to M.W.), the Swiss Society for Muscle Diseases and the Helmut Horten Foundation.

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