Dissecting the role of creatine kinase

The phenotype of 'gene knockout' mice deficient in a creatine kinase isoform sheds new light on the physiological function of the 'phosphocreatine circuit'.

All living organisms require energy to survive and carry out the multitude of tasks that characterize biological activity. Cellular energy demand and supply are generally balanced and tightly regulated for economy and efficiency of energy use. The enzyme creatine kinase plays a key role in the energy metabolism of cells that have intermittently high and fluctuating energy requirements, such as skeletal and cardiac muscle fibres, neurons, electrocytes, photoreceptors and spermatozoa. This is because the reaction catalyzed by creatine kinases — the reversible transfer of the phosphoryl group from phosphocreatine to ADP (Fig. 1) — allows regeneration of the key cellular energy-carrying molecule, ATP. Cells contain a number of different creatine kinase isoforms, and the recent generation of mice homozygous for a germline mutation preventing synthesis of one of the isoforms has confirmed the important part played by creatine kinase in cellular energy metabolism [1].

A phosphocreatine circuit for energy homeostasis

Biochemical fractionation and *in situ* immunolocalization techniques have shown that the different creatine kinase isoforms, once thought to be strictly soluble enzymes, are not distributed evenly in cells. Instead, they are compartmentalized in an isoform-specific fashion: two isoforms, M-CK and B-CK, are cytosolic, and two other isoforms, Mi_a-CK and Mi_b-CK are specifically mitochondrial. These various isoforms of creatine kinase are thought to constitute an intricate energy buffering and transport system connecting sites of high-energy phosphate production (by glycolysis and oxidative phosphorylation) to sites of energy consumption, such as myofibrils and membrane ion pumps (Fig. 2). The specific association of creatine kinase with such sites has allowed the identification and isolation *in vitro* of functionally coupled subcellular microcompartments (for recent reviews see [2,3]).

The mitochondrial creatine kinase isoform, Mi-CK, is located in the mitochondrial intermembrane space, where it is found along the outer surface of the entire inner membrane, and also at sites where the inner and outer membranes are in close proximity (see [3,4] for reviews). At these latter sites, Mi-CK can directly use intramitochondrially produced ATP to generate phosphocreatine, which is exported to the cytosol where it serves as an easily diffusible, energy-storage metabolite (Fig. 2). In contrast to the cytosolic creatine kinase isoforms, which are dimeric, Mi-CK forms highly symmetrical, cube-like octamers [5] and can bind to the periphery of lipid membranes. Most importantly, Mi-CK can mediate contact-site formation between the inner and outer mitochondrial membranes [6] and it is functionally coupled to oxidative phosphorylation by the adenine nucleotide transporter [7,8], which catalyzes ATP/ADP antiport across the inner membrane. This functional coupling has been demonstrated by a number of different experiments, including use of radioactive tracers, showing that mitochondrial

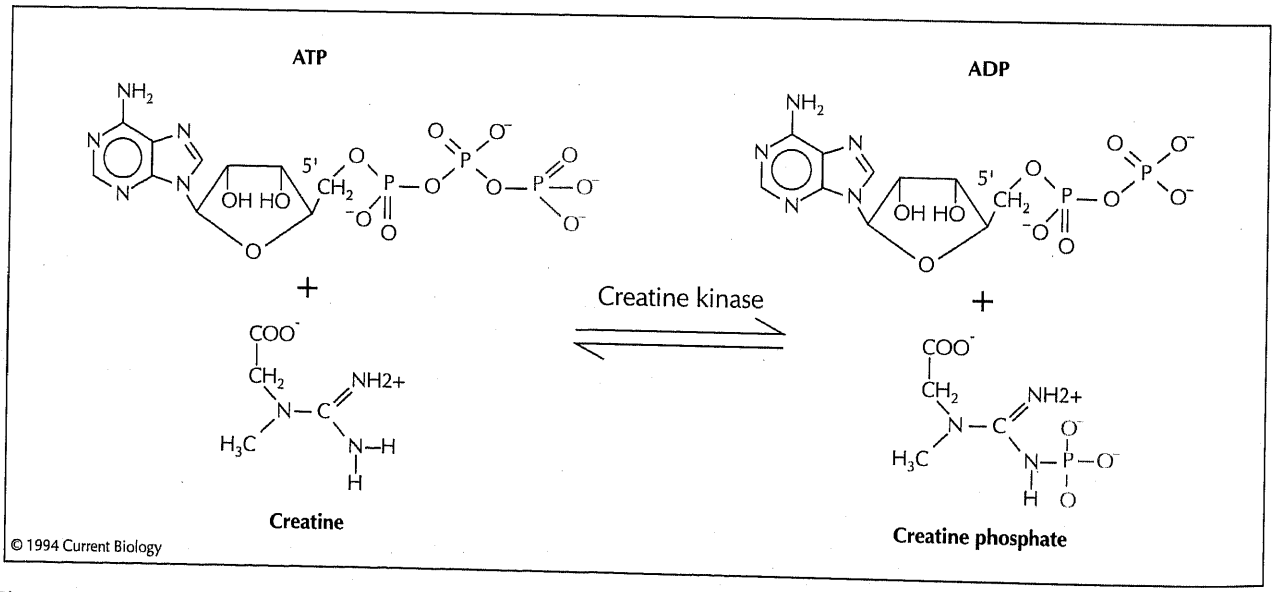

Fig. 1. The transphosphorylation reaction catalyzed by the enzyme creatine kinase.

respiration, and net phosphocreatine production, can be stimulated by addition of extra-mitochondrial creatine, even in the presence of external ATP-regeneration systems and ATP sinks [2,3,7,8].

Coupling also requires transport of creatine and phosphocreatine across the mitochondrial outer membrane: as shown in Figure 2, this occurs through porin molecules, which are in a cation-selective state at inner/outer membrane contact sites, allowing creatine entry into the mitochondrion, and elsewhere are in a high-conductance, anion-selective state, allowing phosphocreatine exit. The cube-like Mi-CK octamer contains a central channel parallel to its four-fold axis [5]. This has led to the suggestion that it acts as a connecting module between adenine nucleotide transporter and porin molecules, forming an efficient, tightly coupled, multienzyme 'energy channel' [9], which combines the directed export of mitochondrial energy equivalents with the interconversion of matrix-generated ATP plus creatine into ADP plus phosphocreatine (Fig. 2). The dynamic equilibrium between Mi-CK octamers and dimers, and the differential, pH-dependent interactions between both dimers and octamers and the mitochondrial inner membrane that have been observed *in vitro* (see Fig. 2) [10], may be important steps at which mitochondrial energetics can be regulated [2–4,10].

The cytosolic creatine kinase isoforms, which in part are associated with specific subcellular compartments or structures [2,11], use phosphocreatine to regenerate

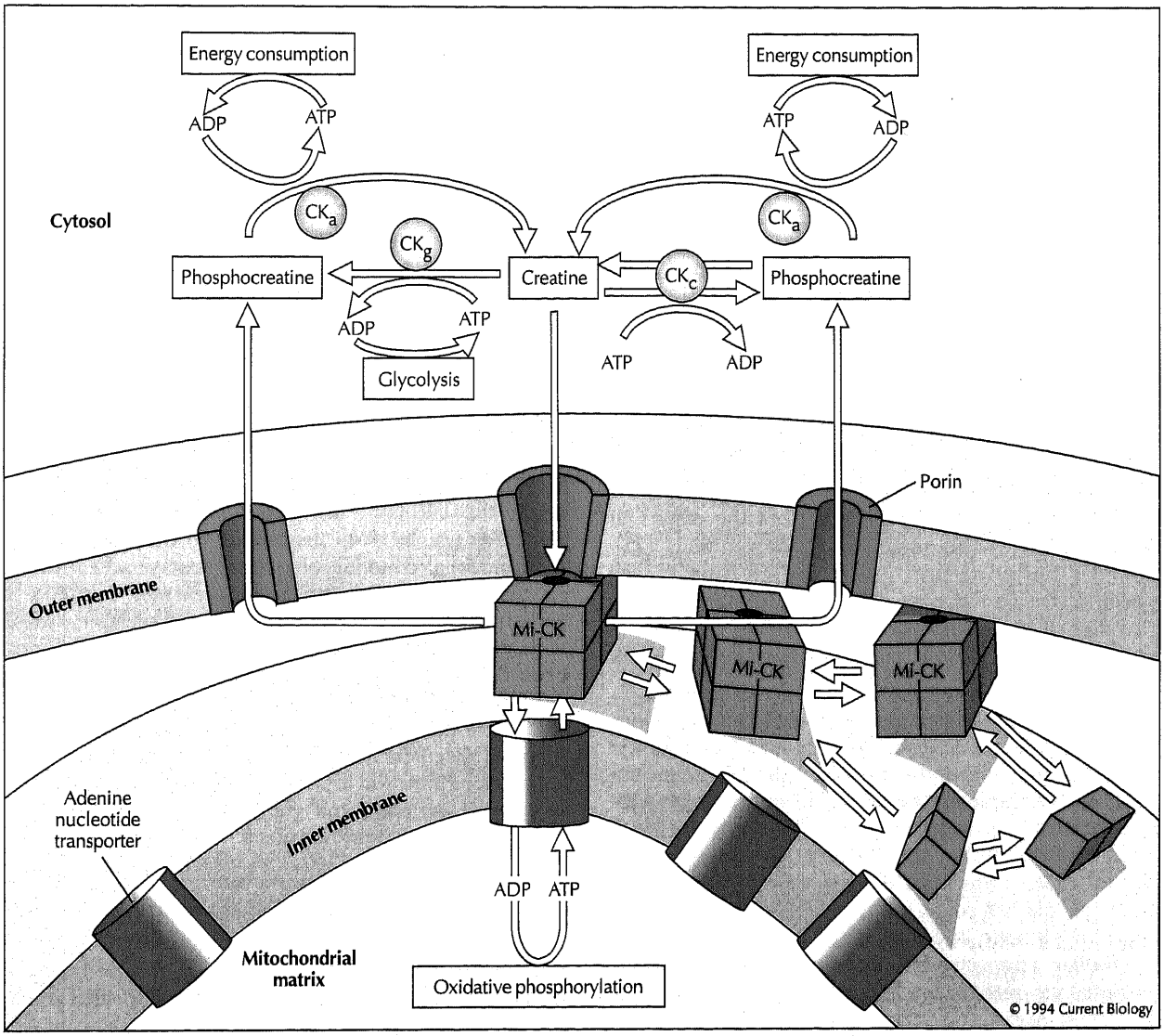

Fig. 2. The phosphocreatine circuit model of intracellular energy homeostasis. Four major compartments of creatine kinase are shown: strictly soluble creatine kinase (CK_c) which catalyzes the free equilibration of the cytosolic ratios of phosphocreatine (PCr)/creatine (Cr) and ATP/ADP; cytosolic creatine kinase (CK_g), which couples glycolysis to phosphocreatine production; 'cytosolic' creatine kinase (CK_a) associated with subcellular sites of high and fluctuating ATP consumption, such as the myofibrils and the sarcoplasmic reticulum in muscle; and mitochondrial creatine kinase (Mi-CK), which is functionally coupled to oxidative phosphorylation. According to this model, small pools of ATP and ADP turn over rapidly and in opposite directions at sites of phosphocreatine production (bottom and middle) and consumption (top); these sites are coupled by creatine kinase and the phosphocreatine/creatine diffusional pathways. Note that the relative pool sizes of phosphocreatine and creatine are much larger than those of the adenine nucleotides. In resting muscle, for example, the concentrations are: PCr, ~20–40 mM; creatine, ~5–15 mM; ATP, ~3–5 mM; ADP, ~10–200 μM. For details of the model see [2,3].

ATP at sites of high energy demand, such as the actomyosin ATPase [12–14] and sarcoplasmic reticulum Ca^{2+} ATPase [15,16] in muscle fibres, and the plasma-membrane Na^+/K^+ ATPase in electrocytes [17]. The localization of creatine kinase at these sites, and the formation of functionally coupled subcellular microcompartments has kinetic and thermodynamic advantages for the workings of the system as a whole [2,3,18]. It has been demonstrated that the myofibrillar actin-activated myosin ATPase and the sarcoplasmic reticulum Ca^{2+} ATPase have privileged access to ATP generated by bound creatine kinase, even in the presence of exogenously added ATP-supplying systems or exogenous ATP traps [16]. Similar studies showing functional coupling between ATP-generating and ATP-consuming systems have been carried out with isolated mitochondria [7,8]. A number of functions have been suggested [2,3] for the communication between mitochondrial and 'cytosolic' creatine kinase isoforms by phosphocreatine and creatine, which have led to the proposal of the 'phosphocreatine shuttle' [7,19] or 'phosphocreatine circuit' models [2,3,16].

Firstly, the phosphocreatine circuit has been suggested to serve as a temporal energy buffer [2,20], keeping ATP and ADP concentrations steady and buffering H^+ ions, thus preventing a rapid fall in the intracellular ATP concentration during cellular work and avoiding intracellular acidification owing to ATP hydrolysis. This suggestion has been fully supported by experiments with transgenic mice expressing the B-CK isoform in liver cells: perfusion of the liver from such animals under anoxic conditions showed a strong buffering effect of the transgene-encoded creatine kinase on intracellular ATP levels and pH [21]. Furthermore, phosphocreatine was shown to protect ATP levels from the effects of fructose load in the same transgenic animals [22]. A similar buffering function of the creatine kinase system in muscle has been demonstrated more recently in studies of 'gene knockout' mice that do not express the muscle-type cytosolic creatine kinase isotype M-CK (see below) [1].

Secondly, it has been suggested that the phosphocreatine circuit has a regulatory role and increases the thermodynamic efficiency of intracellular energy metabolism, for example by keeping the intracellular ADP concentration low and maintaining high ATP/ADP ratios at subcellular sites where it is functionally coupled to ATP-requiring processes [2,18]. Recent evidence for this has come from observations of the functional and kinetic coupling of creatine kinase to the myofibrillar actin-activated myosin ATPase [12–14], the sarcoplasmic reticulum Ca^{2+} ATPase [15,16] and the plasma membrane Na^+/K^+ ATPase [17]. The improvement the creatine kinase system makes in the thermodynamic efficiency of ATP hydrolysis seems especially important for ATPases, such as the sarcoplasmic reticulum Ca^{2+} ATPase, that operate near thermodynamic equilibrium. Furthermore, an important consequence of the combined creatine kinase and ATPase reactions is the net release of inorganic phosphate, which ^{31}P-NMR analysis has shown could be rate-limiting for glycogenolysis and/or glycolysis in muscle in the absence of phosphocreatine hydrolysis [23].

Thirdly, the phosphocreatine circuit is thought also to serve as a spatial energy buffer, or transport system [2,3,8,19,24]. In this role, phosphocreatine is thought to act as an 'energy carrier' connecting sites of energy production, such as the mitochondrial inner membrane, with sites of energy utilization. Mitochondrial creatine kinase would, of course, be expected to play a major part in this process [9]. That creatine kinase has such a role in energy transport is supported by the specific subcellular compartmentalization of the different creatine kinase isoforms in a variety of tissues [2], as well as by the various properties of the Mi-CK isoform discussed above [3]. It is important to note that by speeding up the 'communication' between sites of ATP production and consumption, the phosphocreatine circuit may accelerate and smooth the transitions between different work states; thus, it may dampen oscillations in the concentrations of ADP and ATP and simultaneously reduce the transient times for reaching a new steady-state at a given work load [3].

Although the phosphocreatine circuit model seems well supported by evidence from studies *in vitro*, it has proved very difficult to verify the suggested energy transport role of creatine kinase *in vivo* by ^{31}P-NMR (nuclear magnetic resonance) analysis, and different research teams have produced conflicting results. Thus, whereas the flux through the creatine kinase reaction in the glucose-perfused heart correlates well with cardiac muscle performance [24], which would support the creatine kinase system having a transport function, no such correlation has been reported for skeletal muscle [25]. A clear correlation between creatine kinase-catalyzed flux and brain activity has recently been demonstrated *in vivo* by NMR magnetization transfer [26]. And the functional coupling of creatine kinase and glycolysis, also predicted by the model presented in Figure 2 and suggested by the co-localization of creatine kinase with glycolytic enzymes in muscle [11], has recently been demonstrated directly in anoxic fish muscle using ^{31}P-NMR methods [27]. The results of a number of studies on different systems therefore point to the compartmentalized creatine kinase system having an important general function [2]. But the relative importance of the spatial buffering and energy transport roles of the creatine kinase system remains a matter for discussion.

M-CK gene knockout mice

Wieringa and colleagues [1] have recently succeeded in introducing into the germline of mice a null mutation for the gene encoding the muscle cytosolic creatine kinase isoform, M-CK. Mice homozygous for this mutation completely lack active M-CK enzyme, but still express more-or-less normal levels of the mitochondrial isoform Mi-CK and have normal concentrations of free ATP, phosphocreatine and inorganic phosphate in their resting muscles. Most strikingly, the well-known symmetrical transitions in the concentrations of phosphocreatine and inorganic phosphate during exercise and recovery were similar in mutants and wild-type controls. Nevertheless, on closer analysis of muscle

performance, a clear physiological phenotype became apparent. Muscles of the mutant mice lack the ability to perform burst activity; that is, although the muscles reach a normal initial peak tension, they are unable to maintain that initial peak tension for any length of time. This is exactly what one would expect in the absence of any buffering activity from cytosolic creatine kinase, and thus provides strong additional support for the postulated role of M-CK as an immediate energy buffer for short-term muscle activity.

However, these results raise the intriguing question of how M-CK-deficient mice can still use phosphocreatine as an energy source. The most plausible explanation may be that, under these conditions, the mitochondrial membranes become permeable to phosphocreatine, which could enter the mitochondria and be used to synthesize ATP by Mi-CK. Although the possibility has not been ruled out that some of the Mi-CK isoform — which is encoded by a nuclear gene — is no longer correctly targeted to mitochondria, this explanation seems less likely as only relatively small increases in Mi-CK expression levels were observed in the transgenic animals, and these levels were probably inadequate to account for the maintenance of a creatine kinase reaction in the cytosolic and myofibrillar compartments. Although it might have been expected that the M-CK-deficient mice would compensate by expressing the brain-specific creatine kinase isoform, B-CK, in their muscles, no such misexpression of B-CK was observed. A last possibility is that another, as yet undiscovered, enzyme is responsible for metabolizing phosphocreatine in muscle in the transgenic mice.

The muscles of the mutant mice did, however, show signs of having undergone structural and metabolic adaptations to the absence of M-CK expression: for example, the mitochondrial capacity and glycogen content of their fast muscle fibres were found to be increased. Such adaptations indicate that the absence of M-CK mainly affects fast-twitch muscle fibres, and may change them into slower, oxidative-like fibres. The expansion of the mitochondrial network in fast-twitch fibres of the mutant mice significantly reduces the diffusion distances between mitochondria and myofibrils, suggesting that it is an adaptation to increase the energy transport that can be mediated directly by ATP and ADP. Similar metabolic adaptations and structural plasticity have been observed in rats fed with β-guanidino-proprionic acid (GPA), a creatine analogue that blocks the entry of creatine into muscle tissue [28]. This raises the intriguing question of how a lowered cellular energy status, as in the GPA-fed rat model, or the absence of a key enzyme of energy metabolism, as in the M-CK-deficient mice, can lead to long-term structural and metabolic muscle adaptations.

Even more intriguing is the fact that, unlike in normal mice, no significant fluxes through the creatine kinase reaction can be detected in muscles of M-CK-deficient mice by saturation transfer or inversion transfer ^{31}P-NMR methods, despite the presence of normal levels of Mi-CK [1]. In more recent experiments with mutant mice expressing reduced levels of M-CK, Wieringa and colleagues have found that the NMR-detectable flux through the creatine kinase reaction is highly dependent on the level of M-CK expression. In muscles with less than one third the normal M-CK activity, the flux through the creatine kinase reaction was about 20-times less than normal. The flux suddenly increases to close to normal levels, however, when M-CK activity reaches about half that of wild-type muscle (B. Wieringa and J. van Deursen, personal communication).

One possible explanation for this is that, *in vivo*, ^{31}P-NMR may only be able to detect the flux through truly soluble M-CK, and not that through Mi-CK and through the subset of M-CK molecules associated with subcellular structures, and/or the subset that has access to NMR-invisible substrate pools. It is possible that, when the M-CK level is reduced beyond a certain point, it is all bound to subcellular structures and so the flux through this bound enzyme is ^{31}P-NMR invisible. The latter becomes visible only when the level of soluble CK is high enough to saturate these binding sites. In support of this, in transgenic mice expressing the brain-type isoform B-CK in skeletal muscle, the creatine kinase activity is increased to 150% normal levels and there is a two-fold increase in the energy flux through the creatine kinase reaction [29].

These results imply that the ratio of bound to unbound creatine kinase should be taken into account when interpreting ^{31}P-NMR measurements, and they may explain the failure to find a correlation between creatine kinase reaction fluxes and skeletal muscle activity [24]. These considerations may also shed light on the unexplained peculiarities of ^{31}P-NMR measurements in muscle that have been postulated to arise from the compartmentalization of creatine kinase and/or its substrates in this tissue [2]. Mice expressing variable, subnormal levels of M-CK may therefore be a key experimental system for addressing some of the fundamental questions about ^{31}P-NMR measurements of fluxes through the creatine kinase reaction *in vivo*. Although ^{31}P-NMR is a non-invasive technique, it is still hampered by a relatively low sensitivity and limited spatial resolution (relative to cellular dimensions). In the light of the unusual NMR behaviour of the creatine kinase system in the gene knockout mice, earlier ^{31}P-NMR measurements of creatine kinase fluxes should be interpreted with caution. It is conceivable that, when the complexities of whole organ physiology are better understood, a new era of physiological ^{31}P-NMR studies will begin.

Mice in which the gene for the mitochondrial creatine kinase isoform, Mi-CK, has been 'knocked out' might provide the best model system for addressing the long-debated issue of whether the phosphocreatine circuit has an energy transport or spatial buffering function. In mutant muscle lacking Mi-CK, no short-term effects on burst activity would be expected as long as the cytosolic M-CK buffering capacity remains intact. Such Mi-CK null mutants may, however, be deficient in their long-term muscle performance, unless the muscle again shows a surprising adaptability. One would also predict that knocking-out the gene for the ubiquitous B-CK isoform would have a pronounced effect, especially as this isoform is important for the proper functioning of

many non-muscle cells, such as glia and neurons in the nervous system, photoreceptors in the retina, spermatozoa and so on [2]. Double knockouts of the genes for M-CK and Mi-CK, or B-CK and Mi-CK, would also be expected to cause a severe impairment of muscle function, and in the latter case might cause infertility owing to an impairment of sperm motility, which has been shown crucially to depend on creatine kinase activity [30]. Such double B-CK and Mi-CK gene knockouts may even prove lethal.

A thorough, multidisciplinary study of the M-CK-deficient mice now available, as well as of new mutants that can be expected in the future, will not only be important from a biological point of view, because of the insights it should give into the physiological function of creatine kinase in normal animals, but is also likely to shed new light on how ^{31}P-NMR measurements reflect the complexities of systems *in vivo*, in which enzymes can be sequestered in specific subcellular compartments and substrates are present in distinct pools.

Acknowledgements: I am grateful to B. Wieringa and J. van Deursen for communicating unpublished results. I would also like to thank my collaborators at the IZB for stimulating discussions; special thanks are due to E. Furter-Graves and M. Wyss for carefully reading and discussing the manuscript. This work was supported by SNF grant number 31-33907.92 from the Swiss Society for Muscle Diseases and the H. Horten Foundation.

References

1. Van Deursen J, Heerschap A, Oerlemans F, Ruitenbeek W, Jap P, Ter Laak H, Wieringa B: **Skeletal muscles of mice deficient in muscle creatine kinase lack burst activity.** *Cell* 1993, 74:621–631.
2. Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM: **Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the phosphocreatine circuit for cellular energy homeostasis.** *Biochem J* 1992, 281:21–40.
3. Wyss M, Smeitink J, Wevers R, Wallimann T: **Mitochondrial creatine kinase: a key enzyme of aerobic energy metabolism.** *Biochim Biophys Acta* 1992, 1102:119–166.
4. Brdiczka D: **Contact sites between mitochondrial envelope membranes.** *Biochim Biophys Acta* 1991, 1071:291–321.
5. Schnyder T, Gross H, Winkler HP, Eppenberger HM, Wallimann T: **Structure of the mitochondrial creatine kinase octamer: high resolution shadowing and image averaging of single molecules and formation of linear filaments under specific staining conditions.** *J Cell Biol* 1991, 112:95–101.
6. Rojo M, Hovius R, Demel RA, Nicolay K, Wallimann T: **Mitochondrial creatine kinase mediates contact formation between mitochondrial membranes.** *J Biol Chem* 1991, 266:20290–20295.
7. Jacobus WE: **Respiratory control and the integration of heart high-energy phosphate metabolism by mitochondrial creatine kinase.** *Annu Rev Physiol* 1985, 47:707–725.
8. Saks VA, Kuznetsov AV, Kupriyanov VV, Miceli MV, Jacobus WE: **Creatine kinase of rat heart mitochondria: the demonstration of functional coupling to oxidative phosphorylation in an inner membrane preparation.** *J Biol Chem* 1985, 260:7757–7764.
9. Wyss M, Wallimann T: **Metabolite channeling in aerobic energy metabolism.** *J Theor Biol* 1992, 158:129–132.
10. Schlegel J, Wyss M, Eppenberger HM, Wallimann T: **Functional studies with the octameric and dimeric forms of mitochondrial creatine kinase: differential pH-dependent association of the two oligomeric forms with the inner mitochondrial membrane.** *J Biol Chem* 1990, 265:9221–9227.
11. Wegmann G, Zanolla E, Eppenberger HM, Wallimann T: ***In situ* compartmentation of creatine kinase in intact sarcomeric muscle: the actomyosin overlap zone as a molecular sieve.** *J Muscle Res Cell Motil* 1992, 13:420–435.
12. Wallimann T, Schloesser T, Eppenberger HM: **Function of M-line-bound CK as intramyofibrillar ATP regenerator at the receiving end of the phosphorylcreatine shuttle in muscle.** *J Biol Chem* 1984, 259:5238– 5246.
13. Krause SM, Jacobus WE: **Specific enhancement of the cardiac myofibrillar ATPase by bound creatine kinase.** *J Biol Chem* 1992, 267:2480–2486.
14. Ventura-Clapier R, Mekhfi H, Vassort G: **Role of creatine kinase in force development in chemically skinned rat cardiac muscle.** *J Gen Physiol* 1987, 89:815–837.
15. Rossi AM, Eppenberger HM, Volpe P, Cotrufo R, Wallimann T: **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* 1990, 265:5258–5266.
16. Korge P, Byrd SK, Campbell KB: **Functional coupling between sarcoplasmic-reticulum-bound creatine kinase and Ca^{2+}-ATPase.** *Eur J Biochem* 1993, 213:973–980.
17. Blum H, Balschi JA, Johnson RG: **Coupled *in vivo* activity of creatine phosphokinase and the membrane-bound (Na^+/K^+)-ATPase in the resting and stimulated electric organ of the electric fish *Narcine brasiliensis*.** *J Biol Chem* 1991, 266:10254– 10259.
18. Kammermeier H: **Why do cells need phospho-creatine and a phospho-creatine shuttle?** *J Mol Cell Cardiol* 1987, 19:115–118.
19. Bessman SP, Carpenter CL: **The creatine-creatine phosphate energy shuttle.** *Annu Rev Biochem* 1985, 54:831–862.
20. Meyer RA, Sweeney HL, Kushmerick MJ: **A simple analysis of the phosphocreatine shuttle.** *Am J Physiol* 1984, 246:C365–C377.
21. Miller KR, Halow JM, Koretsky AP: **Transgenic livers expressing creatine kinase are protected during low oxygen: a ^{31}P NMR study.** *Biophys J* 1993, 14:A52.
22. Brosnan JM, Chen L, Wheeler CE, Van Dyke TA, Koretsky AP: **Phosphocreatine protects ATP from a fructose load in transgenic mouse liver expressing creatine kinase.** *Am J Physiol* 1991, 260:C1191–C1200.
23. Meyer RA, Brown TR, Krilowicz BL, Kushmerick MJ: **Phosphagen and intracellular pH changes during contraction of creatine-depleted rat muscle.** *Am J Physiol* 1986, 250:C264–C274.
24. Bittl JA, Ingwall JS: **Reaction rates of creatine kinase and ATP synthesis in the isolated rat heart.** *J Biol Chem* 1985, 240:3512–3517.
25. Brindle KM, Blackledge MJ, Challiss RAJ, Radda GK: **^{31}P NMR magnetization-transfer measurements of ATP turnover during steady-state isometric muscle contraction in the rat hind limb *in vivo*.** *Biochemistry* 1989, 28:4887–4893.
26. Sauter A, Rudin M: **Determination of creatine kinase kinetic parameters in rat brain by NMR-magnetization transfer: correlation with brain function.** *J Biol Chem* 1993, 268:13166–13171.
27. Van Waarde A, Van Den Thillart G, Erkelens C, Addink A, Lugtenburg J: **Functional coupling of glycolysis and phosphocreatine utilization in anoxic fish muscle.** *J Biol Chem* 1990, 265:914–923.
28. Shoubridge EA, Challiss RAJ, Hayes DJ, Radda GK: **Biochemical adaptation in the skeletal muscle of rats depleted of creatine with the substrate analogue β-guanidino-propionic acid.** *Biochem J* 1985, 232:125–131.
29. Brosnan MJ, Sasikala P, Raman LC, Koretsky A: **Alteration of the creatine kinase isoenzyme distribution in transgenic mouse muscle by overexpression of the B subunit.** *Am J Physiol* 1993, 264:C151–C160.
30. Tombes RM, Shapiro BM: **Metabolic channeling: a phosphocreatine shuttle to mediate high energy phosphate transport between sperm mitochondria and tail.** *Cell* 1985, 41:325–334.

Theo Wallimann, Institute for Cell Biology, ETH-Hönggerberg, CH-8093 Zurich, Switzerland.