

## REVIEW ARTICLE

# Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis

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## INTRODUCTION

All the processes involved in growth and metabolism of cells require an input of energy. In living cells, 'production, transport, conversion and utilization of energy' are fundamental processes that are facilitated via metabolic pathways involving a large number of tightly regulated enzyme-catalysed reactions. The regulation of an enzyme system can be accomplished in a number of different ways, e.g. by modulation of its substrate concentrations, by specific regulatory molecules, by post-translational modifications or by subcellular compartmentation. An example of the latter type is creatine kinase (CK), a key enzyme of cellular energetics, representing an enzyme system with a number of isoenzymes, which are in part compartmentalized specifically at those places where energy is 'produced' or 'utilized'. Recent experimental data suggest that CK is located near the sites where the 'real action' occurs, e.g. where force generation by motor proteins, ion-transport across membranes by ion pumps and other ATP-dependent processes take place. The creatine kinase/phosphocreatine (CK/PCr) system seems to play a complex, multi-faceted role in cellular energy homeostasis.

## CREATINE KINASE AND HIGH-ENERGY PHOSPHATE METABOLISM

ATP is the universal energy currency for most of the energy-requiring processes in biological systems (Lehninger, 1982). Excitable cells and tissues, e.g. skeletal and cardiac muscle, brain, photoreceptor cells, spermatozoa and electrocytes, all depend on the immediate availability of vast amounts of energy that may be used in a pulsed or fluctuating manner. Simply increasing intracellular concentrations of ATP for energy storage would represent a bad choice to meet the energy requirements of these cells and tissues, since local [ATP], [ADP] and [AMP], as well as the ATP/ADP ratio, are key regulators influencing many fundamental metabolic processes (Cohen, 1968, 1976; Newsholme & Start, 1973; Atkinson, 1977; Gyulai *et al.*, 1985; Chance *et al.*, 1986; From *et al.*, 1990). Indeed, excitable cells usually contain only 2–5 mM concentrations of ATP, which for example would suffice for a muscle contraction of only a few seconds (Infante & Davies, 1965). Instead, however, large quantities of metabolically inert phosphagens are accumulated in these cells or tissues (Eggleton & Eggleton, 1928; Meyer, 1988). Whereas a variety of

different phosphagens, e.g. phosphoarginine, phospholombicine, phosphoglycocyamine and phosphotaurocyamine, are found in lower phyla (Watts, 1971; Morrison, 1973; Ellington, 1989), phosphocreatine (PCr) is the sole phosphagen in vertebrate and some invertebrate species. Creatine (Cr), synthesized mostly in liver and kidney, but not in muscle, is transported through the blood and taken up by tissues with high energy demands via an active transport system (Fitch *et al.*, 1968). In skeletal muscle, PCr concentrations may reach 20–35 mM or more (Fitch, 1977), depending on the species and the muscle fibre type (Carlson & Wilkie, 1974; Burt *et al.*, 1976; Ackerman *et al.*, 1980), whereas in other excitable tissues like brain, electric organ, smooth muscle, kidney, etc. [PCr] is in the range of 5–10 mM (Iyengar, 1984).

Even though the cellular pools of ATP are rather small, no significant change in overall ATP levels can be detected during activation of excitable tissues (Mommaerts & Wallner, 1967; Seraydarian, 1980), because ATP is continuously and efficiently replenished from the large pool(s) of PCr through the reaction catalysed by creatine kinase (CK; EC 2.7.3.2):



(for reviews see Watts, 1973; Kenyon & Reed, 1983).

In most muscles, the ATP regeneration capacity of CK is very high and considerably exceeds both ATP utilization as well as ATP replenishment by oxidative phosphorylation and glycolysis (McGilvery, 1975). For example, the maximal rate of ATP synthesis by the CK reaction in rat cardiac muscle (30  $\mu\text{mol/s per g}$ ) is much higher than the maximal rate of ATP synthesis by oxidative phosphorylation (2.5  $\mu\text{mol/s per g}$ ) or by *de novo* pathways (0.39  $\mu\text{mol/s per g}$ ) (Bittl & Ingwall, 1985; Ronca & Ronca-Testoni, 1987; Ingwall *et al.*, 1990).

In addition to the regeneration of hydrolysed ATP, the CK system, with CK as a low-threshold ADP sensor ( $K_m$  of MM-CK for ADP is 10–35  $\mu\text{M}$ ) (Matthews *et al.*, 1982; Levin *et al.*, 1990), plays a critical role in preventing a build-up of ADP, especially during transient periods in which energy utilization exceeds energy production. The following metabolite concentrations have been measured in resting muscle: [PCr] = 20–35 mM; [Cr] = 5–10 mM; [ATP] = 3–5 mM; [ $\text{P}_i$ ] = 1.5–2 mM; [free  $\text{Mg}^{2+}$ ] = 3–4 mM; pH = 7.0–7.2 (Ackerman *et al.*, 1980; Chance *et al.*, 1986). Most of the ADP in resting muscle is bound to the F-actin filaments and is metabolically not available to CK for immediate

Abbreviations used: CK, creatine kinase; M- and B-CK refer to the muscle and brain-type subunits of CK, respectively, forming MM-, MB- and BB-CK dimers; Mi-CK stands for mitochondrial CK; ANT, adenine nucleotide translocator, the ATP/ADP carrier; P, outer mitochondrial membrane pore = porin = VDAC; PCr, phosphocreatine; Cr, creatine;  $\text{P}_i$ , inorganic phosphate; GPA,  $\beta$ -guanidinopropionic acid; PKC, protein kinase C.

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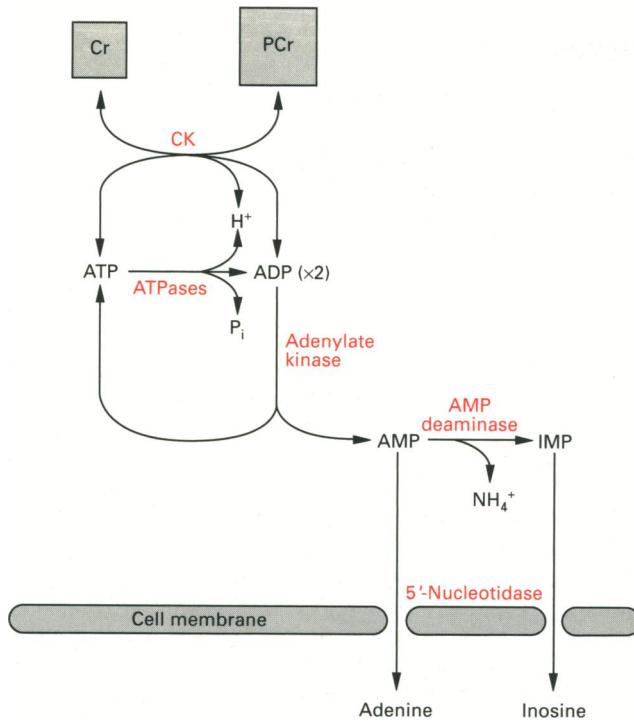


Fig. 1. Creatine kinase involvement in adenine nucleotide metabolism

CK is a fast ATP-regenerating enzyme and a low-threshold sensor for ADP. Two products of cellular ATPases, that is, ADP and H<sup>+</sup>, are substrates of the CK reaction and are consumed during the CK-catalysed regeneration of ATP. Thus, CK is preventing local acidification near cellular ATPases as well as a build-up of ADP. The latter would otherwise lead, after a series of enzymic conversions, to a net loss of cellular adenine nucleotides, which would be deleterious for a cell. ADP (×2): two ADP molecules are converted by adenylate kinase to give one molecule of ATP and AMP each.

rephosphorylation (West *et al.*, 1967). The cytoplasmic concentration of free ADP in resting muscle has been determined by equilibrium calculations as 37  $\mu\text{M}$  (Veech *et al.*, 1979) and was calculated from <sup>31</sup>P-n.m.r. experiments to be in the range of only 1–20  $\mu\text{M}$  (taking CK equilibrium constants from solution kinetics; Bittl & Ingwall, 1985; Meyer *et al.*, 1985; Ackerman *et al.*, 1980). In contrast, chemical analysis revealed values in the range of 100–500  $\mu\text{M}$ , these values being too high due to breakdown of ATP and/or due to liberation of ADP previously bound to subcellular structures, e.g. F-actin (see Chance *et al.*, 1986). A burst of skeletal muscle activity *in vivo* is normally paralleled by the utilization of PCr and the accumulation of free creatine and inorganic phosphate (P<sub>i</sub>), but not with the appearance of equivalent amounts of free ADP (Mommaerts, 1969; Balaban *et al.*, 1986; From *et al.*, 1990). In stimulated muscle, the ADP levels usually rise to only about 100–400  $\mu\text{M}$  or less (Bittl & Ingwall, 1985; Meyer *et al.*, 1985; Chance *et al.*, 1986).

ADP, besides P<sub>i</sub>, Ca<sup>2+</sup>, mitochondrially generated NADH and oxygen supply, is thought to regulate the overall balance between the consumption and oxidative generation of ATP in the intact cell (Gyulai *et al.*, 1985; Chance *et al.*, 1986). For example, mitochondrial respiration is activated by [ADP] in the  $\mu\text{M}$  range (some 20–30  $\mu\text{M}$ -ADP is required for half-maximal activation of mitochondrial ATP synthesis; Chance & Williams, 1955; Chance *et al.*, 1986). However, even though <sup>31</sup>P-n.m.r. experiments with perfused hearts *in vitro* have shown that ADP levels at a given myocardial oxygen consumption rate ( $MV_{O_2}$ ) may vary significantly, depending on the exogenous carbon source in the perfusate, it was concluded that *in vivo* significant changes of

[ADP] and [P<sub>i</sub>] are not essential for the stimulation of mitochondrial oxidative phosphorylation, if the availability of mitochondrially generated NADH is increased to an appropriate extent (From *et al.*, 1990). Indeed, an *in vivo* paced <sup>31</sup>P-n.m.r. study with mammalian heart indicated that the PCr/ATP ratio and thus most likely also the overall [ADP] were surprisingly stable and did not change with increasing work output (see Balaban *et al.*, 1986), although, owing to changes in pH, the relative fractions of MgADP and other ADP species may change somewhat. This is in contrast to experiments with skeletal muscle *in vivo* and with saline-perfused hearts where, in general, changes in the work output correlate with alterations of the PCr/ATP ratio and thus also with changes in [ADP] (Chance *et al.*, 1985; Matthews *et al.*, 1982). Thus, in skeletal muscle, with less overall oxidative capacity for aerobic ATP production, when compared with heart, brain or kidney, the phosphorylation potential is not maintained with increases in work output, e.g. during work jumps. Therefore, in these muscles, ADP and P<sub>i</sub> may play a significant role in the regulation of respiration (Chance *et al.*, 1985) as well as of other metabolic processes such as glycolysis and glycolysis. The picture emerging now is that the control of energy flux between sites of ATP consumption and production is a multistep process with control levels at several sites involving the delivery of reducing equivalents (NADH), oxygen and ATPase hydrolysis products (ADP, P<sub>i</sub>) (for review see Balaban, 1990). In this context, the CK reaction is directly related to the role of ADP and P<sub>i</sub> in the regulation of oxidative phosphorylation and other metabolic processes (Jacobus *et al.*, 1983; Wallimann *et al.*, 1989).

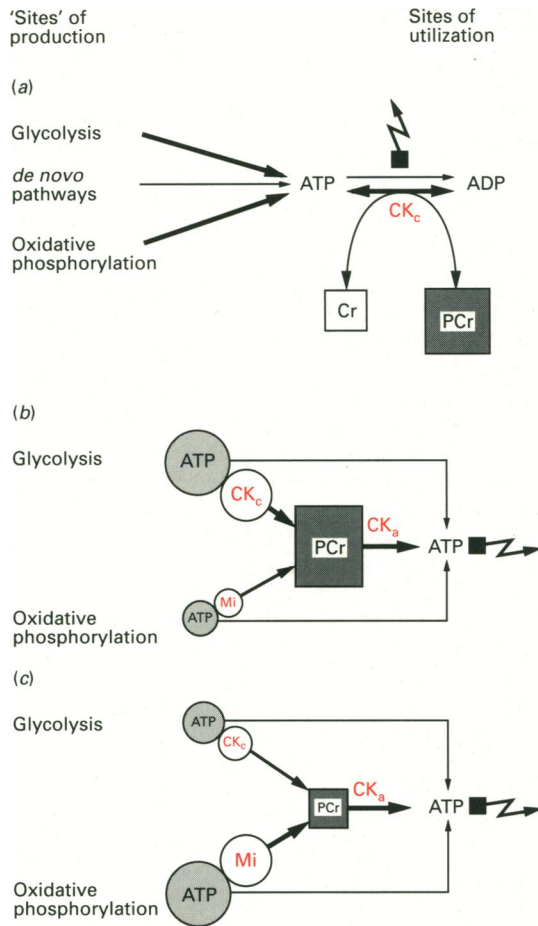
As shown in Fig. 1, a high level of CK can also regulate the adenylate kinase reaction and thus, in effect, conserve the adenine nucleotide pools in the cell (Iyengar, 1984), because if ADP is allowed to accumulate in a cell, e.g. by ischaemia or anoxia (McGilvery & Murray, 1974; Connett, 1988), it is transphosphorylated via adenylate kinase to yield ATP and AMP (Noda, 1973; Hamada & Kubly, 1978). AMP, an inhibitor of adenylate kinase and of metabolic pathways like gluconeogenesis (Uyeda & Racker, 1965; Yamada & Sugi, 1989), is converted into IMP and ammonia by AMP deaminase (Kushmerick & Davies, 1969; Hamada & Kubly, 1978; Lowenstein, 1972) which in muscle is bound to the myofibrils (Ashby *et al.*, 1979) at both ends of the A-band (Cooper & Trinick, 1984). IMP as well as AMP are dephosphorylated by 5'-nucleotidase located on the sarcolemma (Bowditch *et al.*, 1985) to give inosine and adenosine, respectively. Since the sarcolemma is permeable to the latter two compounds, an accumulation of ADP for a prolonged period of time would ultimately lead to a loss of adenine nucleotides (Jennings *et al.*, 1981).

Concluding from these data, there are at least three important metabolic consequences of keeping the cellular ADP levels low by the CK system: (i) CK keeps the free intracellular [ADP] in a range where it may participate in the regulation of mitochondrial respiration, (ii) CK prevents the inactivation of ATPases by rising [ADP], and (iii) CK also prevents a net loss of cellular adenine nucleotides.

### CREATINE KINASE ISOENZYMES

In vertebrate tissues, at least four subunit isoforms of CK are expressed in a tissue-specific manner: two 'cytosolic' forms, M-CK and B-CK (M standing for muscle; B standing for brain), and two mitochondrial Mi-CK isoforms (Mi standing for mitochondrial).

*In vivo*, M-CK and B-CK subunits combine to give the three typical dimeric 'cytosolic' MM-, MB- and BB-CK isoenzymes with an approximate  $M_r$  of 80000–86000 (Eppenberger *et al.*,



**Fig. 2. Models of CK function: temporal and spatial energy buffering**

(a) The CK/PCr system is represented in the classical textbook style as a 'temporal energy buffer' which is disconnected from the three main ATP-regenerating systems, i.e. oxidative phosphorylation, glycolysis and *de novo* ATP synthesis pathways. Here, PCr and Cr provide exclusively parallel diffusion pathways for ATP and ADP, as suggested by the 'facilitated diffusion model' (Meyer *et al.*, 1984). This representation does not take into consideration the subcellular compartmentation of CK, PCr and adenine nucleotides, as well as the functional coupling of CK isoenzymes to glycolysis, oxidative phosphorylation and to various subcellular ATP-consuming processes (cellular ATPases). (b) and (c) The CK/PCr system represented as a 'temporal' as well as a 'spatial' energy buffering or 'energy transport' system. In this model, cytosolic CK is functionally coupled to glycolysis (CK<sub>c</sub>) or to various subcellular sites (CK<sub>s</sub>) with high ATP consumption (black, filled squares with jagged arrow), whereas mitochondrial CK (Mi) is functionally coupled to oxidative phosphorylation. A direct, parallel pathway of diffusion of ATP, produced by glycolysis or oxidative phosphorylation, to subcellular sites of energy consumption is also indicated. (b) depicts schematically the situation in a fast-twitch glycolytic muscle where the 'energy buffering function' of the CK/PCr system is prominent. This is reflected by the large pool size of PCr, the high relative percentage of cytosolic CK (CK<sub>c</sub>) (Yamashita & Yoshioka, 1991), functionally coupled to glycolysis, and the relatively small percentage of Mi-CK [5–10% of total CK in skeletal muscle (Ingwall & Fossel 1983)]. (c) depicts schematically the situation in a slow-twitch oxidative muscle or a cardiac muscle, where the 'energy transport function' of the CK/PCr system is prominent. This is reflected by the relatively small pool size of PCr, the high relative percentage of mitochondrial CK being functionally coupled to oxidative phosphorylation [up to 30% Mi-CK or more of total CK, e.g. in cardiac muscle (Ingwall & Fossel, 1983)] and the relatively small percentage (compared to glycolytic muscle) of cytosolic CK (CK<sub>c</sub>) (Yamashita & Yoshioka, 1991). Abbreviations: CK<sub>c</sub>, 'cytosolic',

1967). Whereas MM-CK is rather specific for differentiated sarcomeric muscle, BB-CK is found in brain and in a variety of other tissues (Eppenberger *et al.*, 1967). Interestingly, during muscle cell differentiation *in vitro* and *in vivo*, a developmental transition from BB-CK via the transitory MB-CK hybrid to the MM-CK homodimer is observed (Perriard *et al.*, 1978; Caravatti *et al.*, 1979; Trask *et al.*, 1988). In chicken, two distinct B-CK subunit subspecies, B<sub>a</sub>- and B<sub>b</sub>-CK, with different isoelectric points were identified (Quest *et al.*, 1989). These B-CK subspecies arise by differential splicing of a unique B-CK gene (Wirz *et al.*, 1990) and dimerize in a tissue-specific manner (Quest *et al.*, 1990a).

Mi-CK is accumulated specifically in mitochondria (Jacobs *et al.*, 1964; Jacobus & Lehninger, 1973; Scholte *et al.*, 1973; DeLuca & Hall, 1980), and two isoforms, striated muscle-specific, 'sarcomeric' Mi<sub>b</sub>-CK (Schlegel *et al.*, 1988a; Hossle *et al.*, 1988; Haas & Strauss, 1990) and so-called 'ubiquitous' Mi<sub>a</sub>-CK (Schlegel *et al.*, 1988b; Hossle *et al.*, 1988; Haas *et al.*, 1989; Wyss *et al.*, 1990) are also expressed in a tissue-specific manner [a and b refer to the isoelectric points of the chicken Mi-CK isoenzymes with Mi<sub>a</sub>-CK being more acidic (pI 8.4–9.0) than Mi<sub>b</sub>-CK (pI 9.3–9.5); Wyss *et al.*, 1990]. Mi<sub>b</sub>- and Mi<sub>a</sub>-CK differ not only in amino acid sequence, although there is a high degree of sequence identity, but also in a number of biochemical and biophysical parameters (Schlegel *et al.*, 1988b; Wyss *et al.*, 1990). In contrast to the cytosolic CK isoenzymes, which are always dimeric, Mi<sub>b</sub>- and Mi<sub>a</sub>-CK were found *in vivo* and *in vitro* to form also octamers with an *M<sub>r</sub>* of approx. 340 000 (Schlegel *et al.*, 1988a; Quemeneur *et al.*, 1988; Belousova *et al.*, 1991; Wyss *et al.*, 1990) composed of four dimers with an *M<sub>r</sub>* of 86 000 as the stable building blocks (Schnyder *et al.*, 1988; Wyss *et al.*, 1990). Some 20 members of the CK isoenzyme family have been sequenced (for references see Babbitt *et al.*, 1986; Hossle *et al.*, 1988; James *et al.*, 1990), and sequence comparisons show extensive homologies both at the DNA and amino acid level between all the phosphagen kinases that have been studied so far.

## MAIN FUNCTIONS OF THE CREATINE KINASE/PHOSPHOCREATINE SYSTEM

The first of the main functions of the CK/PCr system, which is generally accepted and cited in most textbooks, is that of a 'temporal energy buffer' (see Fig. 2a). Besides this function, an increasing body of evidence, discussed below, has been accumulated in favour of a second main function, that of a 'spatial energy buffer' or 'energy transport' system. This idea, for which the term 'CP-shuttle' was coined by Bessman & Geiger (1981), has evolved over the past three decades, and a number of authors, some of them listed here, have made significant contributions towards this metabolic concept (see Figs. 2b, 2c and Fig. 3) (Klingenberg, 1964; Gercken & Schlette, 1968; Naegle, 1970; Gudbjarnason *et al.*, 1970; Bessman, 1972; Turner *et al.*, 1973; Jacobus & Lehninger, 1973; Wallimann, 1975; Seraydarian & Abbott, 1976; Wallimann *et al.*, 1977, 1989; Saks *et al.*, 1978; Bessman & Geiger, 1981; Jacobus *et al.*, 1983; McClellan *et al.*, 1983; Iyengar, 1984; Savabi *et al.*, 1984; Jacobus, 1985a,b; Bessman & Carpenter, 1985; Wallimann & Eppenberger, 1985, 1990; Ventura-Clapier *et al.*, 1987a,b; Meyer, 1988; for a more extensive bibliography, see the reference lists of the above articles and reviews). In this role, PCr would serve as an 'energy carrier', connecting sites of energy production with sites of energy utilization via the subcellularly compartmentalized CK iso-

mostly soluble, CK; CK<sub>s</sub>, CK associated with ATP-consuming processes; Mi, mitochondrial CK; small black squares with jagged arrow, intracellular locations where ATP is hydrolysed.

enzymes. On the basis of kinetic modelling it was suggested that simply 'buffering' the concentration of ATP by PCr and 'facilitated diffusion' are physiologically important, while 'transport', 'shuttling' or 'channelling' of PCr are not (Meyer *et al.*, 1984). Unfortunately, however, the subcellular compartmentation of the CK isoenzymes, as well as that of adenine nucleotides and creatine, were not considered in these model calculations, partly due to a lack of data.

A **third** main function of the CK/PCr system is to prevent a rise in intracellular [free ADP], thus avoiding an inactivation of cellular ATPases and a net loss of adenine nucleotides (Iyengar *et al.*, 1982; Iyengar, 1984).

A **fourth** main function of the CK/PCr system is proton buffering. Since the CK reaction in the direction of ATP regeneration not only utilizes ADP but also protons ( $H^+$ ), which are both products of ATP hydrolysis, an intimate functional coupling of CK with ATPases prevents the local or global acidification of cells which are breaking down high amounts of ATP within a short period of time. This proton-buffering of the CK/PCr system seems especially important in the early phase of severe exercise, before glycogenolysis is activated.

In addition, the release of inorganic phosphate ( $P_i$ ) is another metabolic function of the CK/PCr system. Since net PCr hydrolysis is the major source of  $P_i$ , released at the onset of muscle contraction, and since  $P_i$  is required for activation of glycogenolysis and glycolysis, CK action exerts an indirect regulatory effect for the latter metabolic pathways in muscle types which depend on glycogenolysis (Davuluri *et al.*, 1981). Thus, in these muscles,  $[P_i]$  could become rate-limiting for glycogenolysis in the absence of PCr hydrolysis, a fact supported by  $^{31}P$ -n.m.r. work (see Meyer *et al.*, 1986).

A **fifth** main function of the CK/PCr system is to provide appropriate local ATP/ADP ratios at subcellular sites where CK is functionally coupled to ATP-consuming enzymes or processes. This can be achieved effectively since CK with its low  $K_m$  for ADP, in the range 10–35  $\mu M$  (Watts, 1973; Jacobus & Lehninger, 1973; Saks *et al.*, 1976a; Matthews *et al.*, 1982; Levin *et al.*, 1990), can be considered as a low-threshold ADP sensor. In addition, by maintaining the ATP/ADP ratio high in the vicinity of an ATPase, CK increases the thermodynamic efficiency of ATP hydrolysis ( $\Delta G = \Delta G_{obs.} - RT \cdot \ln[ATP]/[ADP] \cdot [P_i]$ ; Kammermeier, 1987). The same regulatory and thermodynamic aspects also hold true, of course, for CK at the energy-producing side, where the enzyme is coupled to the ATP-generating systems of glycolysis and oxidative phosphorylation (see below) (for review see Wallimann & Eppenberger, 1985, 1990; Wallimann *et al.*, 1989). In this case, CK is minimizing the free energy required for ATP synthesis.

Results concerning the subcellular localization and the relative proportions of the CK isoenzymes as well as the pool sizes of PCr in different tissues of high and fluctuating energy demands led to the suggestion that the CK/PCr system works not only in parallel with ATP and ADP diffusion (Fig. 2a), but, depending on the tissue, also functions as an obligatory link between sites of ATP production and consumption (Figs. 2b and 2c). Taking into account more recent data concerning the structure, localization and function of mitochondrial CK (see below), an integrated 'PCr circuit' model (Fig. 3) was recently proposed (Wallimann *et al.*, 1989; Wallimann & Eppenberger, 1990). In our opinion, the PCr circuit fulfils all five functions of the CK/PCr system mentioned above and represents an intricate 'energy distribution network' connecting intracellular sites of ATP production (mitochondria and glycolysis) with sites of ATP consumption (ATPases) (see Figs. 2 and 3).

The PCr circuit hypothesis is based on and supported by the following principal findings.

### Subcellular and functional compartmentation of CK isoenzymes in sarcomeric muscle

In a variety of tissues, CK isoenzymes are subcellularly compartmentalized in an isoenzyme-specific manner. Fractions of the 'cytosolic isoenzymes' M-CK or B-CK are in part concentrated and specifically localized at sites of energy consumption as well as at sites of energy production, whereas Mi-CK is specifically localized in mitochondria (Figs. 3 and 4) (Wallimann *et al.*, 1977, 1983a, 1985, 1986a,b; for reviews see Saks *et al.*, 1978; Eppenberger *et al.*, 1983; Wallimann & Eppenberger, 1985, 1990; Wallimann *et al.*, 1989). In muscle, the content of PCr and the proportions of CK isoenzymes found in the different subcellular compartments depend strongly on the physiological requirements. In fast-twitch muscle, for example, glycolysis is the main pathway of ATP production. Thus, functional coupling of CK with glycolysis, which is reflected by an increased expression of cytosolic CK ( $CK_c$ ) and the co-localization of this  $CK_c$  with glycolytic enzymes at the I-band (see below), seems advantageous. Together with the very large PCr pool(s) (Newsholme *et al.*, 1978) and the small proportion of Mi-CK (Yamashita & Yoshioka, 1991), this points to an 'energy buffer' function of the CK/PCr system in such muscles (Fig. 2b).

By contrast, in slow-twitch, oxidative muscle or cardiac muscle, where ATP is mainly derived from fatty acid oxidation within mitochondria, a functional coupling of Mi-CK with oxidative phosphorylation, which is reflected by the expression of Mi-CK at relatively high levels (Ingwall *et al.*, 1980), seems advantageous. Together with the rather small PCr pool(s) and the low levels of cytosolic  $CK_c$  (Yamashita & Yoshioka, 1991), this points to an 'energy transport' function of the CK/PCr system (Fig. 2c). In both kinds of muscles, some proportion of 'cytosolic' CK ( $CK_a$ ) is associated with sites of ATP consumption where  $CK_a$  is functionally coupled to ATP-requiring processes (Figs. 2b and 2c). Parallel pathways of energy flux directly via ATP and ADP, as suggested by the 'facilitated diffusion model' (Meyer *et al.*, 1984) are also indicated by arrows. Note the schematic representation of relative proportions of CK isoenzymes and PCr pools in Figs. 2(b) and 2(c).

### Functional coupling of CK with ATP-requiring processes in muscle

In muscle, 5–10 % of 'cytosolic' CK, depending on the muscle fibre-type and the procedures used for preparation of myofibrils, are localized at the myofibrillar M-band (Turner *et al.*, 1973; Wallimann *et al.*, 1977, 1978, 1983a,b) where CK is functionally coupled to the myofibrillar, actin-activated  $Mg^{2+}$ -ATPase (Wallimann *et al.*, 1984). This localization was shown to be isoenzyme-specific for MM-CK (Wallimann *et al.*, 1983a), whereby the C-terminal half of M-CK seems to contain the major epitope(s) responsible for binding of the enzyme to the M-band (Schäfer & Perriard, 1988). The amount of M-band CK is sufficient to regenerate the ATP hydrolysed during muscle contraction even if the myofibrillar  $Mg^{2+}$ -ATPase is working under optimal conditions *in vitro* (Wallimann *et al.*, 1984). The localization of CK at the M-band between the two acto-myosin overlap zones seems favourable. During muscle contraction, both the interdigitation of thin filaments into the thick filament lattice, as well as the movement of myosin heads, seem to push the reaction products of the myofibrillar  $Mg^{2+}$ -ATPase (ADP,  $H^+$  and  $P_i$ ), which are in part also the substrates for CK (ADP and  $H^+$ ) (Fig. 1), from both A-band halves towards the M-band (Wallimann & Eppenberger, 1985), as could be directly demonstrated recently (Winegrad *et al.*, 1989). Furthermore, in an *in vitro* system, substrate channelling and coupled activity between CK bound to frog heart muscle filaments and myosin

ATPase was also shown (Arrio-Dupont, 1988). The data concerning the functional role of myofibrillar CK discussed above are in accord with physiological studies showing the dependence of relaxation and tension development of intact muscle or skinned muscle fibres on myofibrillar-bound CK and on the PCr content (Spande & Schottelius, 1970; Ventura-Clapier *et al.*, 1987a,b; Seraydarian, 1980; Hoerter *et al.*, 1991). In contrast to the findings by Otsu *et al.* (1989), we did not find MM-CK evenly distributed over the entire A-band, but localized the enzyme specifically at the M-band of skeletal and cardiac muscle (see Wallimann & Eppenberger, 1985; Wallimann *et al.*, 1989) and within the I-band (Wegmann *et al.*, 1987, 1991b). It seems that MM-CK and other even smaller-sized 'soluble' enzymes, like adenylate kinase, or glycolytic enzymes, like aldolase, are mostly excluded from the acto-myosin overlap region of the A-band, and are concentrated together at the I-band, where they form multi-enzyme complexes (see Brooks & Storey, 1988) loosely associated with the thin filaments (Arnold & Pette, 1970; Wegmann *et al.*, 1991b; see below). Besides its enzymic function as an ATP regenerator, the M-line-bound MM-CK also serves a structural role in the myofibrillar architecture by forming the so-called m4 and m4' m-bridges (Wallimann *et al.*, 1983b; Strehler *et al.*, 1983) which interlink and possibly also position the thick filaments within the myofibril (Luther *et al.*, 1981; for review see Wallimann & Eppenberger, 1985).

In muscle, additional portions of MM-CK have been found to be specifically associated with the sarcoplasmic reticulum (SR; Baskin & Deamer, 1970; Khan *et al.*, 1972; Levitsky *et al.*, 1977; Sharov *et al.*, 1977; Elizarova *et al.*, 1987; Rossi *et al.*, 1990) where CK is functionally coupled to the ATP-dependent Ca<sup>2+</sup>-pump (Levitsky *et al.*, 1977, 1978) supporting ATP-driven Ca<sup>2+</sup>-uptake into isolated SR vesicles (Levitsky *et al.*, 1978; Rossi *et al.*, 1990). The specific association of CK with the SR and T-tubule system, confirmed also by *in situ* immuno-gold labelling of permeabilized muscle or by immuno-gold labelling of isolated SR membrane vesicles (Rossi *et al.*, 1990), was resistant to high-salt and low-salt/EDTA treatment, indicating a rather strong anchorage of fractions of CK either directly with the SR membrane and/or an association of CK with SR proteins (Rossi *et al.*, 1990). A direct interaction of cytosolic CK isoenzymes with membranes was recently demonstrated with model membranes (Rojo *et al.*, 1991a). CK associated with the SR membrane is thought to be important not only for providing ATP, but also for regulating local ATP/ADP ratios (Rossi *et al.*, 1990). Recently, it was shown that inositol 1,4,5-trisphosphate receptor-mediated Ca<sup>2+</sup>-efflux from the SR is allosterically regulated by external [ATP] (Ferris *et al.*, 1990), so that an additional regulatory role of SR-bound MM-CK for Ca<sup>2+</sup>-release, as suggested earlier (Rossi *et al.*, 1990), may be postulated. To conclude, these results indicate that CK is critically involved in regulation of ion fluxes taking place during excitation-contraction coupling.

Relatively small, but significant, amounts of MM-CK have also been found at the sarcolemma membrane (Sharov *et al.*, 1977; Jockers-Wretou *et al.*, 1977; Elizarova *et al.*, 1987) where CK is functionally coupled to the ATP-dependent Na<sup>+</sup>/K<sup>+</sup>-pump (Saks *et al.*, 1977; Grosse *et al.*, 1980; see Wallimann *et al.*, 1989).

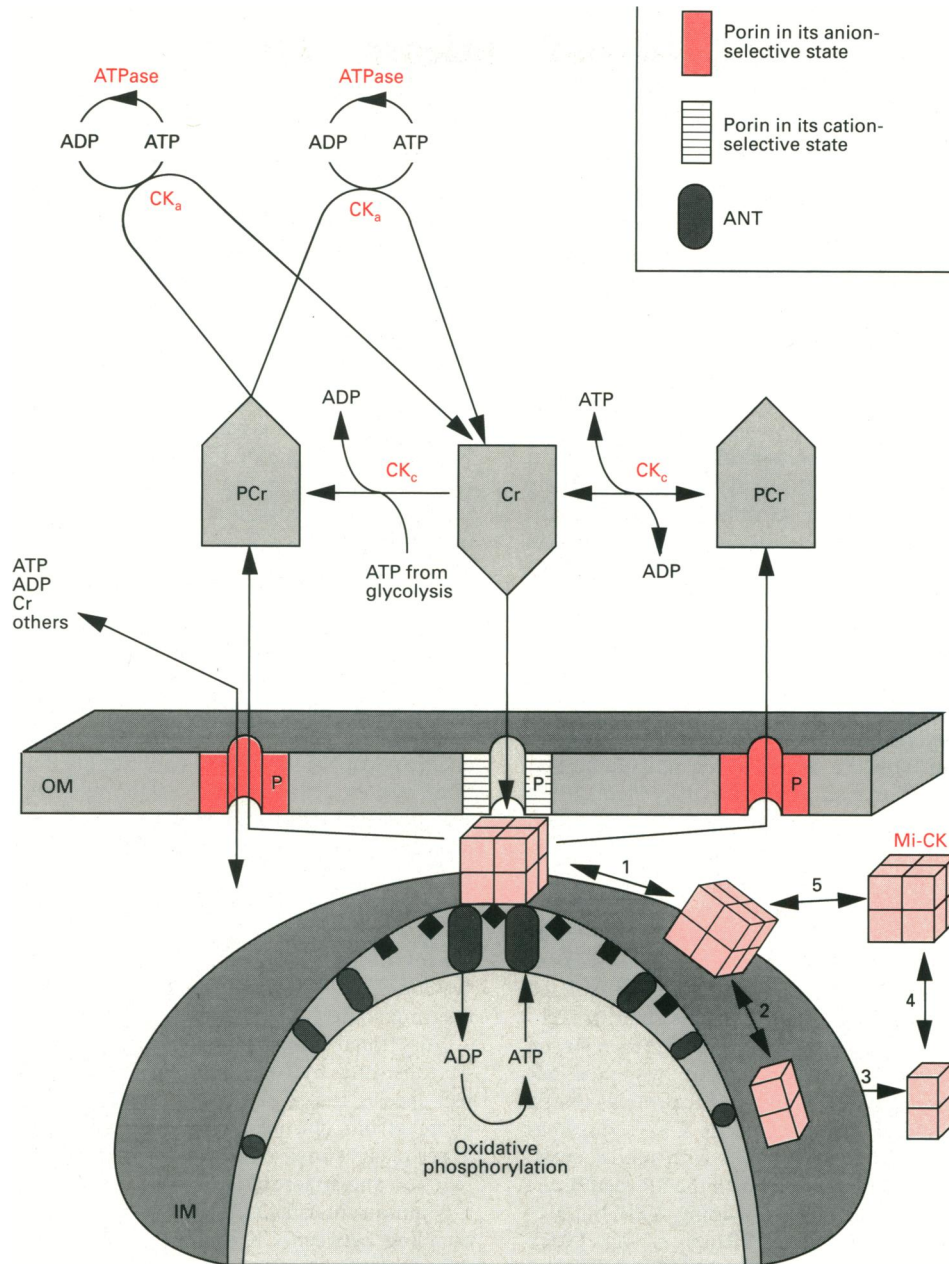
#### Functional coupling of CK with glycogenolysis and glycolysis

Net hydrolysis of PCr is the major source of inorganic phosphate (P<sub>i</sub>) released at the onset of contraction. Glycogenolysis and glycolysis require P<sub>i</sub> for activation. Therefore, P<sub>i</sub>-release indirectly mediated by CK may itself be an important function of the CK system. A functional coupling of CK with glycogenolysis and glycolysis is supported by several lines of

evidence. In muscle, the glycolytic enzymes, forming a so-called 'glycolytic complex' (Brooks & Storey, 1988), are located at the I-band where they are loosely associated with the thin filaments (Arnold & Pette, 1970; Bronstein & Knoll, 1981). Incidentally, most of the soluble MM-CK is also specifically localized at the I-band (Wallimann *et al.*, 1989; Wegmann *et al.*, 1991b) and is eluted from skinned muscle fibres together with most of the glycolytic enzymes (Maughan & Wegner, 1989). In addition, the amount of soluble MM-CK correlates with the glycolytic potential of a muscle (Wallimann *et al.*, 1984), e.g. the highest concentrations of PCr and CK are found in those skeletal muscles with the greatest anaerobic potential (Newsholme *et al.*, 1978). The ATP produced by glycolysis upon stimulation of a fast-twitch glycolytic muscle is not accumulated as such, but is immediately and efficiently transphosphorylated by CK into PCr to replenish the PCr pool(s) and to maintain a high PCr/Cr ratio in the cell (Fig. 2b). This was also shown by studies with a reconstituted glycolytic system of muscle, indicating that Cr is efficiently phosphorylated to PCr via CK coupled to anaerobic glycolysis (Scopes, 1973). A coupling of CK to glycolysis is also supported by the finding that a depletion of [PCr] together with lowered ATP levels occurred in an animal model with defective muscle glycolysis (Brumback *et al.*, 1983).

Additionally, in heart cells, which in general depend on an oxidative metabolism, energy derived from glycolysis can also contribute, at least to some extent, to the maintenance of high-energy phosphate levels and contractility if oxidative phosphorylation of these cells is inhibited (Doorey & Barry, 1983). However, the glycolytic flux and the functional coupling of CK with glycolysis, seen at low PCr/Cr ratios, is suppressed under normoxic conditions (when respiration is normal and the PCr/Cr ratio is high; Kupriyanov *et al.*, 1980), the explanation being a limited availability to glycolysis of ADP, which in heart is kept at low levels by mitochondrial respiration and by the CK system. Thus CK, as a low-threshold sensor for ADP, is also exerting some control over the glycolytic flux.

Functional coupling of glycolysis and PCr utilization was also demonstrated by <sup>31</sup>P-n.m.r. experiments with anoxic muscle of a fish species that can survive anaerobic conditions, deriving its energy from glycogenolysis (Van Waarde *et al.*, 1990). These experiments showed that at elevated levels of free ADP, the CK reaction and anaerobic glycolysis are coupled via protons (H<sup>+</sup>) as a common intermediate (Hochachka & Mommsen, 1983). The coupling between CK and glycolysis seems to disappear upon reoxygenation, when mitochondrial respiration causes a rapid drop in [free ADP] (Van Waarde *et al.*, 1990). Coupling of CK and anaerobic glycolysis by protons is plausible, since the CK reaction is strongly pH-dependent (Wallimann *et al.*, 1984), and protons are consumed by the CK reaction in the direction of ATP regeneration (Fig. 1). A drop in intracellular pH of muscle observed *in vivo* from 7.2 (at rest) to 6.7 (upon prolonged stimulation) (Burt *et al.*, 1976) was shown *in vitro* to activate MM-CK by 50% in the direction of ATP regeneration, the reason for this being that the pH optimum of MM-CK in this direction is between 6.5 and 6.7 (Wallimann *et al.*, 1984). Furthermore, a functional coupling of pyruvate kinase (PK) and CK was demonstrated *in vitro* by <sup>31</sup>P-n.m.r., showing that the formation of a 'diazyme' complex of the two proteins via their common substrate ATP increases the net flux of substrates through the two kinetically coupled reactions (Dillon & Clark, 1990). These findings fit into the concept that the formation of multienzyme complexes as well as the 'metabolic coupling' of sequential (Sreere, 1987) and non-sequential enzymic reactions, like the PK-ATP-CK system described above (Dillon & Clark, 1990), play a fundamental role in metabolic regulation (Srivastava & Bernhard, 1986, 1987).



**Fig. 3. The PCr circuit model for tissues with high and fluctuating energy requirements**

A complex regulated network is presented where the CK/PCr system is proposed to fulfil important functions (i) for 'temporal energy buffering', (ii) for 'spatial energy buffering', directing intracellular 'energy flux', (iii) for keeping cellular [free ADP] low and preventing a net loss of adenine nucleotides, (iv) for removing protons and thus preventing acidification within a cell, and for indirectly stimulating glycogenolysis and glycolysis by  $P_i$  released as a consequence of net PCr hydrolysis, (v) for controlling local ATP/ADP ratios and thus also for increasing the thermodynamic efficiency of ATP hydrolysis. ATP is derived from the two major synthetic pathways, oxidative phosphorylation (shown at the lower half) and glycogenolysis or glycolysis (shown at the left upper middle). Four major compartments for CK isoenzymes are indicated: (i) 'cytosolic' CK ( $CK_a$ ), functionally coupled to glycolysis on the producing side of the PCr circuit (at the left upper middle); (ii) mitochondrial CK (Mi-CK), functionally coupled to oxidative phosphorylation also on the producing side of the PCr circuit (at the lower half); (iii) 'cytosolic' CK, specifically associated ( $CK_b$ ) with subcellular structures at sites of high and fluctuating ATP utilization on the receiving side of the PCr circuit where it is functionally coupled to the corresponding ATPases (at top left) and (iv) strictly soluble cytosolic CK ( $CK_c$ ) freely equilibrating PCr/Cr and ATP/ADP pools of the cytosol (at the right upper middle). In sarcomeric muscle, the functional compartment where  $CK_c$  is coupled with glycolysis is at the I-band where MM-CK is co-localized with the glycolytic enzymes (Wegmann *et al.*, 1987, 1991b; Wallimann *et al.*, 1989). As represented schematically, the relative pool sizes of phosphocreatine (PCr) and creatine (Cr) are much larger than those of the adenine nucleotides. On the mitochondrial side, the functional and structural compartmentation of Mi-CK octamers at the inner/outer mitochondrial membrane 'energy-transfer contact sites' is depicted. The Mi-CK octamer is shown here to interact with adenine nucleotide translocators (ANT) of the inner mitochondrial membrane (IM), possibly via an involvement of cardiolipin ( $\blacklozenge$ ), and with voltage-gated ion-selective pores (P) of the outer membrane (OM), to form an efficient multienzyme trans-membrane 'energy-channelling complex'. It is important to note that the Mi-CK octamer is able to induce and stabilize close contacts between inner and outer mitochondrial membranes (Rojo *et al.*, 1991b). According to this model, ATP generated by oxidative phosphorylation, after transport through the inner mitochondrial membrane by the adenine nucleotide translocator (ANT) in exchange for ADP, is transphosphorylated by Mi-CK to give PCr which then, as a net product of oxidative phosphorylation, leaves the mitochondrion just beyond the contact sites through porin (P) of the outer membrane in its high-conductance, anion-selective state. In contrast, creatine (Cr) is entering through porin within the contact sites, where the porin channel is thought to be in its cation-selective state due to capacitive coupling of the inner

### MITOCHONDRIAL CK: LOCALIZATION, STRUCTURE AND FUNCTIONAL COUPLING TO OXIDATIVE PHOSPHORYLATION

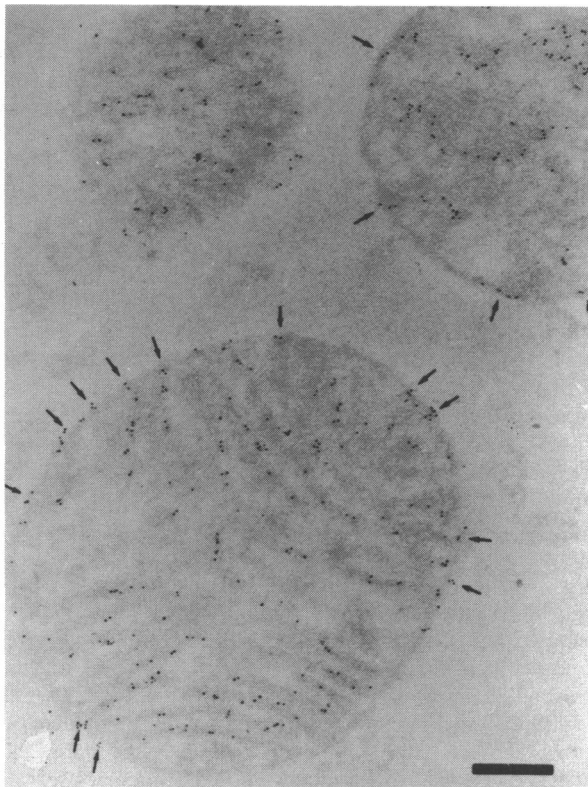
Interestingly, in cells with high energy demands, cytosolic CK isoenzymes are generally co-expressed with Mi-CK. Mitochondrial CK isoenzymes, like cytosolic CKs, are also encoded for by nuclear genes, but are imported into mitochondria, as revealed by the typical tripartite presequence (Haas & Strauss, 1990), by the appearance of a short-lived precursor protein (Perryman *et al.*, 1983; Haas *et al.*, 1989), and by subcellular fractionation (Jacobs *et al.*, 1964). Efficient functional coupling between the Mi-CK reaction and oxidative phosphorylation has been established by biochemical, kinetic and thermodynamic methods as well as by radioisotope analysis (Bessman & Fonyo, 1966; Jacobus & Lehninger, 1973; Saks *et al.*, 1976a,b; Yang *et al.*, 1977; Altschuld & Brierley, 1977; Saks *et al.*, 1980, 1984, 1985, 1986; DeFuria *et al.*, 1980; Moreadith & Jacobus, 1982; Erickson-Viitanen *et al.*, 1982a,b; Gellerich & Saks, 1982; Barbour *et al.*, 1984a; Jacobus, 1985a; Jacobus & Diffley, 1986; Gellerich *et al.*, 1987, 1989; Kuznetsov *et al.*, 1989; Kottke *et al.*, 1991). Thus, Mi-CK seems to have privileged access to mitochondrial matrix-generated ATP presented by the adenine nucleotide translocator (ANT) (for reviews see Bessman & Carpenter, 1985; Jacobus *et al.*, 1983; Jacobus, 1985a) (see Figs. 2b, 2c and 3). Even though no unambiguous proof for a direct physical interaction of Mi-CK with ANT, the most prominent integral membrane protein of the inner mitochondrial membrane (Klingenberg, 1979), has yet been reported, an association of Mi-CK with the inner membrane itself is supported by several lines of evidence: (i) subfractionation studies have shown that Mi-CK is associated with the outer face of the inner membrane (Scholte *et al.*, 1973; Jacobus & Lehninger, 1973). (ii) The enzyme, present mostly in its octameric form in mitochondria (Marcillat *et al.*, 1987; Schlegel *et al.*, 1988a, 1990; Quemeneur *et al.*, 1988; Lipskaya & Trofimova, 1989a,b), remains bound to hypo-osmotically swollen mitochondria or mitoplasts (Scholte, 1973; Vial *et al.*, 1979; Font *et al.*, 1981, 1987; Brooks & Suelter, 1987a) and can be released from mitoplasts with phosphate predominantly in its octameric form (Schlegel *et al.*, 1988a, 1990; Wyss *et al.*, 1990). Mi-CK can be rebound to extracted mitoplasts (Lipskaya *et al.*, 1980; Hall & DeLuca, 1980; Marcillat *et al.*, 1987), whereby at slightly alkaline pH, Mi-CK octamers rebound preferentially over dimers (Schlegel *et al.*, 1990). Once Mi-CK dimers are bound to the mitoplasts, they can undergo octamerization most probably facilitated by lateral diffusion on the inner membrane (Schlegel *et al.*, 1990; see Fig. 3). The octamer/dimer ratio of Mi-CK is influenced *in vitro* by substrates, pH, ionic

strength and protein concentration (Lipskaya *et al.*, 1985; Marcillat *et al.*, 1987; Schlegel *et al.*, 1988a). When MgADP, creatine and nitrate, known to induce a transition-state-analogue complex of CK (Milner-White & Watts, 1971) as well as to dissociate octameric Mi-CK *in vitro* into stable dimers (Schlegel *et al.*, 1988a, 1990), are added to mitoplasts, Mi-CK is concomitantly released from the membranes (Marcillat *et al.*, 1987). (iii) Mi-CK has been localized *in situ* by immuno-gold methods along the mitochondrial cristae membranes (Schlegel *et al.*, 1988a; Wegmann *et al.*, 1991a) (Fig. 4). (iv) Mi-CK interacts with cardiolipin vesicles (Schlame & Augustin, 1985; Müller *et al.*, 1985; Cheneval & Carafoli, 1988). Cardiolipin is a prominent phospholipid component and in fact the only negatively charged phospholipid of inner mitochondrial membranes (Hovius *et al.*, 1990). (v) Recently, it was shown that Mi-CK is able to interact with model membranes of different phospholipid composition, as well as with monolayers of inner mitochondrial membrane phospholipids (Rojo *et al.*, 1991a) (Fig. 5).

Apart from an interaction of Mi-CK with the inner mitochondrial membrane, some recent experimental evidence shows that Mi-CK also interacts with the outer membrane: (i) Mi-CK, mainly in its octameric form as in intact mitochondria, is enriched in isolated boundary membrane contact site fractions together with other peripheral kinases like hexokinase and nucleoside diphosphate kinase (Adams *et al.*, 1989; Kottke *et al.*, 1991). Mi-CK within the contact sites displayed significant latency in its enzymic activity to externally added substrates or inhibitors (Kottke *et al.*, 1991). (ii) Immuno-gold labelling often revealed a clustering of Mi-CK at those places where the inner approaches the outer membrane (Schlegel *et al.*, 1988a; Wegmann *et al.*, 1991a) (see Fig. 4). (iii) In *in situ* histochemical experiments, CK-specific stain product was trapped at contact sites between inner and outer mitochondrial membranes (Biermans *et al.*, 1989, 1990). Both the extent of contact site formation and the area occupied by accumulated stain product significantly increased or decreased as a function of metabolic stimulation or inhibition, respectively (Biermans *et al.*, 1990). This may indicate that the formation and extent of inner-outer membrane contacts are dynamic and depend on the metabolic state of the mitochondrion, as suggested on the basis of freeze-fracturing electron micrographs of intact mitochondria (Knoll & Brdiczka, 1983; Bücheler *et al.*, 1991). (iv) In several reports, it was stressed that the outer mitochondrial membrane and in particular the mitochondrial pore protein play a significant role for the 'dynamic compartmentation' of adenine nucleotides in the mitochondrial intermembrane space (Erickson-Viitanen *et al.*, 1982b; Gellerich *et al.*, 1987, 1989; Brooks & Suelter, 1987b) and for the transfer of PCr to the cytosol (Brdiczka, 1990; Kottke *et al.*, 1991). The

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and outer membrane being in close apposition at these sites (Benz *et al.*, 1990; Brdiczka, 1991; see the text). The possible regulatory aspects of the dynamic octamer/dimer equilibrium of Mi-CK, while bound to the inner membrane (2) or in solution in the intermembrane space (4), and the differential pH-dependent rebinding of the two oligomeric Mi-CK species to the inner mitochondrial membrane (3, dimers; 5, octamers; 2, octamerization of membrane-bound dimers), all observed *in vitro* (Schlegel *et al.*, 1990), are also shown schematically and are indicated by numbers. Although the Mi-CK octamers are distributed over the entire inner membrane, an accumulation of Mi-CK was noted at the inner/outer membrane contacts (1). It should be stressed here that by the schematic representation it is *not* intended to suggest that Mi-CK octamers are only enzymically active at the contacts, but rather that functional coupling of Mi-CK with ATP/ADP translocators (ANT) and with outer mitochondrial membrane pores (P), facilitating the transport of matrix-generated ATP through the inner membrane, the transphosphorylation of this ATP into PCr as well as the export of PCr with concomitant import of Cr, may be most efficient at these sites. At the receiving side of the PCr circuit, CK<sub>a</sub>, being functionally coupled to a variety of cellular ATPases, e.g. to ATP-requiring cellular motor proteins, and being associated with various subcellular microcompartments, e.g. with myofibrils and membranes, is also included in this model (ATPases, top left). For example, CK<sub>a</sub> is associated with myofibrils, sarcoplasmic reticulum and plasma membranes where the enzyme has been shown to be functionally coupled to the myofibrillar ATPase, the Ca<sup>2+</sup>-ATPase (Wallimann *et al.*, 1984; Rossi *et al.*, 1990) and the Na<sup>+</sup>/K<sup>+</sup>-ATPase (Blum *et al.*, 1991), respectively. At these sites, as well as at the producing sites of the PCr circuit, only small pools of ATP are turned over with no need for ATP or ADP diffusion over long distances. Instead, CK isoenzymes are specifically compartmentalized at these locations, and the 'transport of energy' is facilitated by the PCr circuit via PCr and Cr, the former representing not only an inert 'energy buffer' but also a 'transport form' of high-energy phosphates (for details see the text). The PCr circuit model presented here emphasizes the functional coupling with ATP production and consumption and stresses the diffusion pathways of PCr and Cr. However, it is not intended to exclude parallel pathways for ATP and ADP.



**Fig. 4. Localization of Mi-CK in mitochondria**

Immunogold labelling of chicken photoreceptor cell mitochondria after freeze-substitution and low-temperature embedding, using a specific rabbit anti-(chicken Mi-CK) antibody, followed by goat anti-(rabbit IgG) conjugated to 5 nm colloidal gold particles (for experimental details see Wegmann *et al.*, 1991). Note that Mi-CK is located along the cristae membranes, and is also concentrated peripherally at places where inner and outer mitochondrial membranes are in close proximity, presumably at contact sites (as indicated by arrows). Bar = 250 nm. (Reprinted from Wegmann *et al.*, 1991a, with copyright permission from Springer-Verlag, Heidelberg, Germany.)

translocation of adenine nucleotides, PCr and Cr across the outer mitochondrial membrane is suggested to be restricted to the pore-forming protein or 'porin' (Roos *et al.*, 1982), also known as VDAC (voltage-dependent anion carrier) (Colombini, 1979). Porin can assume two different states: a high-conductance, anion-selective and a low-conductance, cation-selective state (Benz *et al.*, 1990; Brdiczka, 1990). It was suggested that the latter, which does not permit passage of negatively charged compounds like ADP, ATP and PCr (see Fig. 3), is induced at mitochondrial contact sites by 'capacitive coupling' between the inner membrane, known to display a membrane potential, and the outer membrane. The pores beyond the contacts would remain anion-selective, since there, the separation distance between the two membranes is too large to allow 'capacitive coupling' (Benz *et al.*, 1990) (see also Fig. 3). As a consequence, creatine (Cr) would pass the cation-selective pores at the contacts, whereas PCr would leave the intermembrane compartment through the anion-selective pores just beyond the contacts (Fig. 3). All these notions are supported by the findings that, although the outer membrane was disrupted by digitonin, a specific pore-blocking polyanion reversibly inhibited Mi-CK activity by blocking the transport of externally added adenine nucleotides through the outer membrane pore (Kottke *et al.*, 1991) and that Mi-CK was inaccessible to negatively charged CK inhibitors and to the

substrate PCr, if these compounds were added externally (Kuznetsov *et al.*, 1989; Kottke *et al.*, 1991). (v) Recently, Mi-CK was shown to interact not only with monolayers of inner membrane phospholipids, but also with such of outer membrane phospholipid mixtures (Rojo *et al.*, 1991a). Furthermore, Mi-CK octamers were able to induce close contacts between a monolayer of spread outer membranes and inner membrane vesicles (Nicolay *et al.*, 1990; Rojo *et al.*, 1991b; see Fig. 5). Compared to octamers, Mi-CK dimers were significantly less effective in inducing close contacts. In addition, neither cytosolic CK isoenzymes (MM- and BB-CK) nor mitochondrial adenylate kinase, also a basic intermembrane protein like Mi-CK, showed any tendency to induce membrane contacts (Rojo *et al.*, 1991b) (see Fig. 5). (vi) Data on the structure of the Mi-CK octamer itself (Fig. 6) also explain the property of Mi-CK octamers to link inner (IM) and outer membranes (OM) together (Fig. 5), exactly as one would expect it to happen at mitochondrial energy transfer contact sites (Fig. 3). The octamer is composed of four identical dimers forming an indentation or channel in the midst of the cube-like molecule, as revealed by various electron microscopical techniques (DeLuca & Hall, 1980; Schnyder *et al.*, 1988, 1991a,b; Belousova *et al.*, 1991; Winkler *et al.*, 1991) (see Fig. 6), as well as by X-ray analysis of Mi-CK crystals (Schnyder *et al.*, 1990). These results point out that the cube-like octamer has two identical top and bottom faces, differing from the four side faces, and that the funnel-like central depressions of the top and bottom face are connected by a central channel (Schnyder *et al.*, 1991a) (Fig. 6). Thus, it seems very likely that the Mi-CK octamer via its two opposing top and bottom faces can interact simultaneously with the inner and outer mitochondrial membranes, and it is tempting to speculate that the translocation of substrates and products occurs through the central channel of the Mi-CK octamer (Wallimann *et al.*, 1989; Schlegel *et al.*, 1990; Brdiczka, 1991) (see Fig. 3). It may even be hypothesized that octameric Mi-CK, tetrameric ATP/ADP translocator (ANT) (Vignais *et al.*, 1989) and oligomeric porin (P) (Krause *et al.*, 1986; Linden & Gellerfors, 1983) form an extended, highly organized multienzyme 'energy transport channel' through the inner and outer membrane at the contact sites (Fig. 3).

It should be stressed here that by the schematic representation in Fig. 3, it is not intended to suggest that Mi-CK octamers are only enzymically active at the mitochondrial contact sites, but rather that functional coupling of Mi-CK with ATP/ADP translocators and with porin facilitates the transphosphorylation of matrix-generated ATP into PCr, its export out of the mitochondrion, and the import of Cr into the mitochondrion.

Up to now, the regulatory cycle concerning the Mi-CK octamer/dimer equilibrium and the differential interaction of octamers versus dimers with the inner mitochondrial membrane were investigated *in vitro* (Lipskaya *et al.*, 1985; Schlegel *et al.*, 1990; Rojo *et al.*, 1991a,b) (schematically depicted in Fig. 3). The estimated *in vivo* concentration of Mi-CK in the intermembrane space would clearly favour an 'all-octamer situation' of Mi-CK (see Schlegel *et al.*, 1990). However, since the octamer/dimer equilibrium as well as the interaction of octamers and dimers with mitochondrial membranes are sensitive to substrates and products of the CK and the ATP/ADP translocator reactions as well as to pH, and since a certain proportion of dimeric Mi-CK is always found when Mi-CK is released from mitochondria, such dynamic cycles are likely to exist also *in vivo*.

#### **The function of Mi-CK in conservation of the mitochondrial phosphorylation potential**

The specific location of Mi-CK between the mitochondrial envelope membranes raises the question of the metabolic advantage of such a localization. At first glance, it would appear



more efficient to produce the PCr inside the matrix compartment and then to export it as such. However, one has to consider that the intramitochondrial phosphorylation potential is significantly lower than that in the cytosol (8 kcal or 33.5 kJ/mol in the mitochondria versus 12 kcal or 50.2 kJ/mol in the cytosol). In spite of the low ATP/ADP quotient in the matrix [approx. 0.2–2.0 or 6–12, as measured in rat liver mitochondria by chemical determination (Soboll *et al.*, 1978) or by  $^{31}\text{P}$ -n.m.r. (Ogawa & Lee, 1982), respectively], mitochondria are still able to contribute to the high extramitochondrial phosphorylation potential because of the asymmetric, electrogenic exchange of ATP versus ADP by the adenine nucleotide translocator (ANT) (Klingenberg *et al.*, 1969). Thus, due to the significantly higher ATP/ADP quotient at the outer side of the inner membrane compared to that in the matrix compartment, the free energy of ATP hydrolysis increases during the export of ATP through the inner mitochondrial membrane. According to Klingenberg (Klingenberg *et al.*, 1969; Klingenberg, 1980), the inner membrane potential can generate ATP/ADP quotients of 15–30 which are comparable to those determined in the cytosol of liver cells (Soboll *et al.*, 1978), but much lower than in muscle where the same parameter is in the range of 100–200 (Hebisch *et al.*, 1986). Klingenberg investigated the efficiency of ATP export in competitive *in vitro* experiments without an active transphosphorylation system, like CK, present (Klingenberg, 1980). However, it is obvious that a direct coupling of CK to the inner membrane and to the ANT itself could significantly improve the efficiency of the mitochondrial energy exchange system since the free energy of hydrolysis of ATP, just exported from the matrix, is directly conserved in the form of PCr, which is not a substrate for the translocator. Thus Mi-CK is minimizing the free energy required for ATP synthesis in the mitochondria. Concomitantly with this transphosphorylation of ATP to give PCr, the ANT gets saturated with ADP and is ready for another ATP/ADP antiport reaction. Due to the facts that the free energy of hydrolysis of ATP exported by the ANT is kept at a very high level and that the intermembrane PCr and Cr levels probably do not equilibrate with the extramitochondrial ones, Mi-CK is able to phosphorylate Cr even at a high cytosolic phosphorylation potential. A further advantage for efficient PCr production by the mitochondria might result from the location of Mi-CK just behind the cation-selective outer membrane pore in the contacts (Benz *et al.*, 1990; Kottke *et al.*, 1991) (Fig. 3). In this shielded position, Mi-CK would be unable to sense external PCr/Cr levels and could produce PCr in spite of a high phosphorylation potential in the cytosol (Brdiczka, 1991).

#### Correlation between relative amounts of mitochondrial CK and energy fluxes through the CK reaction measured by $^{31}\text{P}$ -n.m.r.

It is accepted that in living muscle the CK reaction velocity can be several-fold larger than the rates of ATP hydrolysis and ATP synthesis by oxidative phosphorylation. This overcapacity of the CK system is probably one of the reasons why a positive correlation between oxygen consumption of heart muscle and the CK reaction velocity could only recently be verified by  $^{31}\text{P}$ -n.m.r. techniques applied to perfused heart (Kupriyanov *et al.*, 1984; Perry *et al.*, 1988), by direct biochemical measurements (Mahler, 1985) and by model calculations (Connett, 1988) (Figs. 2b and 2c).

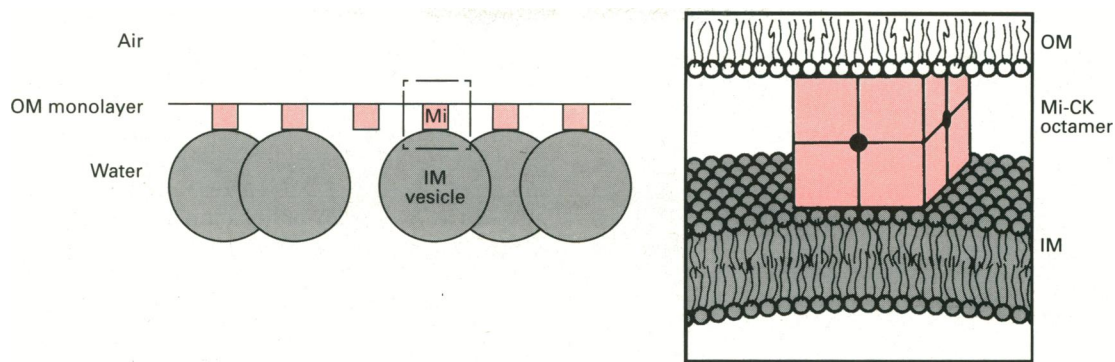
The correlation factor between oxygen consumption and CK reaction flux in cardiac muscle depends on the relative proportion of Mi-CK in the tissue (Perry *et al.*, 1988). Mi-CK is absent from embryonic rat, mouse and rabbit cardiac muscle (Hall & DeLuca, 1975; Ingwall *et al.*, 1980; Ingwall & Fossel, 1983) and is gradually expressed in increasing amounts during postnatal development of these insessorial animals (McAuliffe *et al.*, 1989)

whereby the 'transport function' of Mi-CK becomes increasingly important. During this period, the flux of energy through the CK/PCr system was found to increase in parallel with the developmental accumulation of Mi-CK (Perry *et al.*, 1988), with relative proportions of Mi-CK reaching the 20–30% level of total CK activity observed in adult heart at about 3–4 weeks after birth (Ingwall & Fossel, 1983). In large animals like sheep (Ingwall *et al.*, 1980) or in autophagous birds like chickens, which have to walk and feed on their own shortly after birth, maturation of the CK system occurs around birth or hatching, respectively (Hall & DeLuca, 1976; Wegmann *et al.*, 1991a).

Interestingly, during chronic stimulation of skeletal muscle (Schmitt & Pette, 1985) or training for long distance running (Apple & Rogers, 1986), the conversion of fast-twitch to slow-twitch muscle fibres is accompanied by a significant decrease in total CK activity as well as in PCr pool size. At the same time, however, a severalfold elevation of Mi-CK activity was noticed. Therefore, the absolute amount of Mi-CK correlates with the oxidative potential of the corresponding muscle fibres (Wallimann *et al.*, 1989; Yamashita & Yoshioka, 1991). This, in our opinion, is a strong argument for two of the main functions proposed for the PCr circuit, that is, for the 'energy buffering' function which is more prominent in glycolytic, fast-twitch muscles with high levels of cytosolic CK and large PCr pools (Wallimann & Eppenberger, 1985) (Fig. 2b), and for the 'energy transport' or 'channelling' function which is more pronounced in oxidative slow-twitch and cardiac muscle, both containing relatively high amounts of Mi-CK (approx. 12 and 15–25% Mi-CK of total CK, respectively, compared to glycolytic muscle with only 4%) (Ingwall & Fossel, 1983; Wallimann *et al.*, 1989; Wallimann & Eppenberger, 1990; Yamashita & Yoshioka, 1991) (Fig. 2c).

#### Cellular response to creatine depletion: Mi-CK and long-term metabolic adaptation to a low cellular energy status

Feeding of animals with the creatine analogue  $\beta$ -guanidino-propionic acid (GPA) leads to quite dramatic metabolic changes in their skeletal muscles. For example, a 90% depletion of PCr, a 20–50% decrease of total CK activity and a decrease in glycolytic potential concomitant with an increased oxidative capacity have been described (Fitch *et al.*, 1974; Shoubridge *et al.*, 1985a). Since there was no significant difference in muscle performance in such animals, these results were taken as an argument that neither PCr nor the activity of CK are critical for aerobic metabolism (Shoubridge & Radda, 1984). However, the PCr levels in animals 'intoxicated' with GPA were still in the  $K_m$  range of myofibrillar M-band CK (1.67 mM; Saks *et al.*, 1976a) and larger than the PCr concentration differential or gradient (0.1–0.2 mM) necessary to support maximal energy flux from mitochondria to the myofibrils (Jacobus, 1985b). Furthermore, long-term compensatory metabolic adaptations take place in muscles of GPA-fed animals (Shoubridge *et al.*, 1985b), and metabolic fibre-type transformations and alterations in isoenzyme expression were noted as a consequence of a chronically low PCr and low ATP status. The extent of adaptation, specifically the increase in mitochondrial content, the ability of the muscles of animals fed with GPA to activate aerobic metabolism more effectively than in control animals, and the metabolic shift towards an endurance, low-fatigue type of muscle (Shoubridge & Radda, 1984) argue for the importance of the PCr circuit still operating in GPA-treated animals. Most significant is the recent finding that, in contrast to earlier results with skeletal muscle (Meyer *et al.*, 1986), in cardiac muscle, GPA-phosphate, although a poorly metabolized PCr analogue (Chevli & Fitch, 1979), is used by CK during work jumps and can therefore buffer transitions between work states quite efficiently (Conley &



**Fig. 5. Mediation by Mi-CK of contact formation between mitochondrial membranes**

A monolayer of outer mitochondrial membrane (OM) or OM phospholipids was spread at the air-water interface, and Mi-CK molecules (shaded squares) injected into the water subphase. Mi-CK penetrated into the monolayers (shown schematically on the left) as evidenced by an increase in surface pressure (Rojo *et al.*, 1991a). After washing of the subphase, radioactively labelled inner mitochondrial membrane vesicles (IM) or IM phospholipid vesicles were injected into the subphase, and the increase in surface radioactivity, caused by Mi-CK-mediated binding of vesicles to the surface monolayer, was monitored before and after washing of the subphase (Rojo *et al.*, 1991b). The membrane contact formation observed (shown at higher magnification on the right) was specific for Mi-CK and occurred neither with cytosolic CK isoenzymes nor with a number of cationic mitochondrial intermembrane space enzymes. Most interestingly, the two oligomeric forms of Mi-CK, octamer and dimer, differed in their ability to induce intermembrane adhesion, the octamer being more potent in this respect (Rojo *et al.*, 1991b). The structure of the cube-like Mi-CK octamer with two similar top and bottom faces and four distinct side faces (Schnyder *et al.*, 1990, 1991a,b; see Fig. 6) is consistent with its role in mediating the formation of contacts between two membranes and with the localization of Mi-CK octamers at mitochondrial contact sites. We propose that Mi-CK octamers behave similarly in the intermembrane space of mitochondria *in vivo*, i.e. that they interact simultaneously with the inner and outer membrane at contact sites and possibly also with apposed cristae membranes (Rojo *et al.*, 1991b; see the text and Fig. 3).

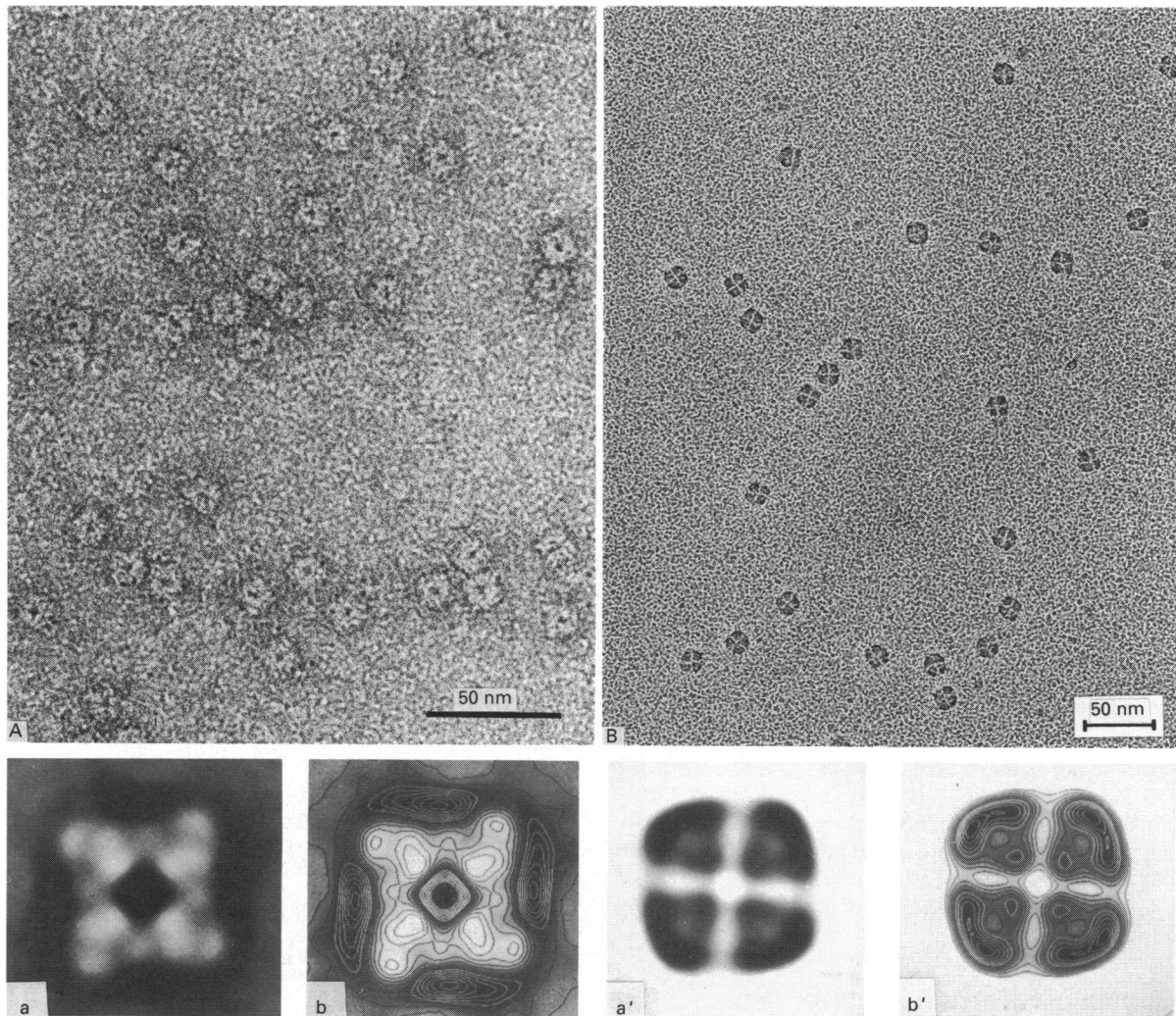
Kushmerick, 1990). In addition, the most recent study with EPA-fed rats suggests that PCr-depleted hearts display fundamental biochemical, physiological and thermodynamic differences compared with control hearts (Zweier *et al.*, 1991).

As part of the adaptations described, large cylindrical mitochondria with paracrystalline inclusions, highly enriched in Mi-CK, were found in adult rat cardiomyocytes cultured in a creatine-free medium (Eppenberger-Eberhardt *et al.*, 1991). The inclusions and Mi-CK accumulation therein disappeared or reappeared simply by the addition to the culture medium of 20 mM-creatine or 10 mM-GPA, respectively. It is our opinion that these cultured heart cells compensate for the low intracellular energy (low total Cr) status in two ways: an accumulation of Mi-CK would (i) increase the catalytic ( $V_{max}/K_m$ ) efficiency of PCr production even at substantially lower cytosolic total Cr levels than normal, and it would (ii) also increase the 'energy transport' function of the PCr circuit. Such an interpretation is in line with a  $^{31}\text{P}$ -n.m.r. study using a normoxic heart model, perfused with 2-deoxy-D-glucose and insulin (Hoerter *et al.*, 1988). Although in this case the PCr levels were depleted by as much as 85–90% of the original and ATP was no longer measurable by n.m.r., surprisingly, the hearts were still beating with 65% of their original systolic pressure. Obviously, under normoxic conditions, when the oxygen supply is normal, the energy transport function of the CK/PCr system was still sufficiently operational (compare Figs. 2b and 2c) to maintain a reasonably high 'energy flux' despite the very low levels of high-energy phosphates (low 'energy buffering capacity') observed after deoxyglucose and insulin administration. This contrasts to the hypoxic situation, in which heart performance was seen to correlate with PCr levels, i.e. if the resupply of ATP by oxidative phosphorylation was inhibited and therefore the 'energy transport' function of the CK/PCr system diminished, heart performance dropped very quickly due to exhaustion of the cellular PCr pool(s) (Hoerter *et al.*, 1988). Experiments measuring PCr depletion and  $\text{O}_2$  uptake in frog sartorius muscle (Mahler, 1985) fully agree with this interpretation, suggesting that respiration in muscle may be rate-limited by the production of ADP via Mi-CK.

#### DIFFUSION LIMITATIONS OF ADENINE NUCLEOTIDES

As far as the energy transport function of the CK/PCr system is concerned, PCr and Cr seem to be better suited as 'transport molecules' than ATP and ADP, since the former are available at much higher concentrations within cells with high, fluctuating energy requirements. In addition, PCr and Cr are smaller compounds with either no or lower negative charge compared to the adenine nucleotides. The suitability of PCr and Cr as 'transport molecules' was recently confirmed by non-invasive  $^{31}\text{P}$ -pulsed gradient n.m.r. measurements *in vivo*, indicating that the mean diffusion lengths of PCr and Cr (57 and 37  $\mu\text{m}$ ) are significantly higher than those of ADP and ATP (1.8 and 22  $\mu\text{m}$ ) (Yoshizaki *et al.*, 1990). These results also confirmed that, *in vivo*, ADP, with a mean diffusion length of 1.8  $\mu\text{m}$ , is clearly the most diffusion-limited of all the above metabolites (Jacobus, 1985b; Mahler, 1985; Yoshizaki *et al.*, 1990), but its diffusiveness may still be sufficient for energy transport in small-sized cells or in structurally specialized, larger-sized cells. For example, certain insect flight muscles, capable of extremely high aerobic energy fluxes, do not seem to contain mitochondrial phosphagen kinases (Schneider *et al.*, 1989; Ellington & Hines, 1991). In these specialized muscles, rows of densely packed mitochondria are lined up in close apposition to individual myofibrils (Smith, 1966) in such a way that diffusion distances from mitochondria to myofibrils are very small. However, in muscle fibres with large diameters and with mostly subsarcolemmal mitochondria, rather large diffusion distances (10–100  $\mu\text{m}$ ) have to be overcome. There, the ADP diffusion rates are 1–2 orders of magnitude below those required for the highest rates of oxidative metabolism (Kammermeier, 1987). In addition, at a certain distance from any mitochondrion (2–3  $\mu\text{m}$ ), the limitation of ADP diffusion would induce a drop in the free energy of ATP hydrolysis by 4–9 kJ/mol, that is, below the values critical for the regular function of the respective ATP-dependent processes (Mainwood & Rakusan, 1982).

The problem of 'energy supply' and 'transport' between sites



**Fig. 6. High-resolution electron micrographs of Mi-CK octamers**

(A) Projections of individual Mi-CK octamers after negative staining with uranyl acetate. (a) is the corresponding averaged image at a resolution of approximately 2 nm and (b) represents a grey-level contour representation of (a). Note the four-fold symmetry of the Mi-CK octamer having a side length of approximately 10 nm and a central cavity of 2.5 nm in diameter, filled by negative stain. (B) Surface relief structure of Mi-CK after high-resolution heavy metal (Ta/W) shadowing at  $-250^{\circ}\text{C}$  under ultra-high vacuum (for experimental details see Schnyder *et al.*, 1991a, and Winkler *et al.*, 1991). The corresponding averaged pictures with a resolution of approximately 2.5 nm are displayed in (a') and in (b') as grey-level contour representation. Note again the four-fold symmetry of the Mi-CK octamer (10 nm  $\times$  10 nm), the cross-like surface indentation indicating the boundaries of the four dimers, as well as the central funnel-like access leading into the interior of the molecule.

of ATP production and sites of ATP utilization, including the thermodynamic consequences stated above, seems to be especially pronounced in highly polar cells with spatially compartmentalized CK isoenzymes, e.g. in photoreceptor cells (Wallimann *et al.*, 1986a; Hemmer *et al.*, 1989; Wegmann *et al.*, 1991a) and in spermatozoa (Tombes & Shapiro, 1985; Wallimann *et al.*, 1986b; Tombes *et al.*, 1987). In these cells, diffusion limitations of adenine nucleotides are likely to occur, since the sites of energy production (mitochondria containing Mi-CK) and the sites of energy consumption (with a specific association of B-CK; Quest & Shapiro, 1991) are separated by large distances, e.g. by  $50\ \mu\text{m}$  from the inner segment to the outer segment of photoreceptors and by up to  $100\ \mu\text{m}$  from the midpiece to the distal end of the sperm tail (see below). The properties of the CK/PCr system seem well suited to overcome the diffusional limitations in these polar cells by utilizing PCr and Cr as mediators between mitochondria and sites of energy utilization.

#### INTRACELLULAR COMPARTMENTATION OF HIGH-ENERGY PHOSPHATES

Since the CK isoenzymes are compartmentalized within a cell, and since the enzymic CK reaction works in opposite directions at energy-producing and energy-consuming sites, it is likely that the substrates and products of the CK reaction are also compartmentalized to some extent within a cell, at least locally. Recent work indeed indicates that within a cell, certain low- $M_r$  metabolites like adenine nucleotides may not be evenly distributed, but may be microcompartmented even without separation by membranes (Jones, 1986; Miller & Horowitz, 1986, and references therein). This may be especially relevant for muscle with its highly structured contractile apparatus with locally changing Donnan potentials (Bartels & Elliott, 1985). Biochemical as well as recent  $^{31}\text{P}$ -n.m.r. studies on muscle are clearly in favour of distinct intracellular ATP pools

(Gudbjarnason *et al.*, 1970; Nunnally & Hollis, 1979; Ugurbil *et al.*, 1979; McClellan *et al.*, 1983; Barbour *et al.*, 1984b; Saks *et al.*, 1984; Koretsky *et al.*, 1985, 1986, 1990; Zahler *et al.*, 1987; Gellerich *et al.*, 1987; Ishida & Paul, 1989; Brindle *et al.*, 1990) as well as creatine pools (Savabi, 1988), for example in mitochondria and myofibrils. In addition, some of the ATP pools are invisible by  $^{31}\text{P}$ -n.m.r. (Toyo-oka *et al.*, 1986; Murphy *et al.*, 1988). Although the n.m.r. visibility of cellular ATP is still a matter of debate, an elegant study by Hutson *et al.* (1989) suggests that at least in isolated mitochondria, all matrix ATP is n.m.r.-visible *in vitro*. The 'dynamic compartmentation' of adenine nucleotides in the mitochondrial intermembrane space (Gellerich *et al.*, 1987, 1989) is also relevant in this respect, since intermembrane [ADP] was shown to depend on the mitochondrial respiratory rate (Gellerich *et al.*, 1989), even though overall [ADP] may be rather constant when measured globally by  $^{31}\text{P}$ -n.m.r. (Balaban *et al.*, 1986; Balaban, 1990; From *et al.*, 1990).

These facts may all complicate the calculation of [free ADP] by  $^{31}\text{P}$ -n.m.r. methods, because the determination of the latter value relies on the actually measured overall concentrations of ATP, Cr and PCr and on the equilibrium constant of the CK reaction taken from enzyme kinetics in solution. Indeed, in a very recent study it was shown that the reaction velocities of CK *in vivo*, measured by  $^{31}\text{P}$ -n.m.r. magnetization transfer, do not always agree with the reaction velocities predicted from solution kinetics (McAuliffe *et al.*, 1991), even if the CK flux measurements were corrected for the exchange of  $\gamma\text{-P}_i$  from ATP to  $\text{P}_i$ , imposing a problem in these measurements (Ugurbil *et al.*, 1986). Thus the actual ADP concentrations, especially on a subcellular level, may differ significantly due to subcellular compartmentation of CK and of its metabolites. This compartmentation should be taken into account and the consequences thereof borne in mind if experimental data, resulting from the measurement of global events, are to be evaluated.

#### SUBCELLULAR COMPARTMENTATION AND FUNCTION OF CK ISOENZYMES IN TISSUES OTHER THAN SARCOMERIC MUSCLE

Multiple CK isoenzymes, cellular and subcellular compartmentation, isoenzyme-specific subcellular localization and functional compartmentation of CK were also observed in a number of tissues other than sarcomeric muscle, e.g. in spermatozoa (Tombs & Shapiro, 1985; Wallimann *et al.*, 1986b), retina photoreceptor cells (Wallimann *et al.*, 1986a; Hemmer *et al.*, 1989; Wegmann *et al.*, 1991a), electrocytes (Barrantes *et al.*, 1983a,b, 1985; Wallimann *et al.*, 1985), visceral and vascular smooth muscles (Ishida *et al.*, 1991), intestinal epithelial cells (Keller & Gordon, 1991), kidney (Ikeda, 1988) and brain (Hamburg *et al.*, 1990).

#### CK compartmentation and function in spermatozoa: metabolite channelling via a PCr shuttle between mitochondria and tail

Two isoforms of CK, brain-type BB-CK and mitochondrial Mi-CK, were identified in spermatozoa from rooster and man. Immunolocalization studies showed that the two CK isoforms are spatially segregated, that is, BB-CK was found in the sperm tail while Mi-CK was confined to the mitochondria within the mid-piece (Wallimann *et al.*, 1986b). Similarly, two main CK isoforms have been characterized in sea urchin sperm; a head-form, presumably corresponding to the mitochondrial CK isoform, as well as a unique tail-form with an  $M_r$  of 145000 (Tombs & Shapiro, 1985, 1987), which during evolution has originated from a gene triplication (Wotho *et al.*, 1990). The tail CK occurs free in the cytosol as well as bound to the sperm tail plasma membrane (Quest & Shapiro, 1991) and to axonemal

microtubules (Tombs *et al.*, 1988), thereby leading to compartmentation of the enzyme within the sperm flagellum. In addition, a subspecies of tail CK, presumably corresponding to the 10% of tail CK associated with the plasma membrane *in vivo*, was shown to bind readily to phospholipid liposomes and detergent micelles *in vitro* (Quest & Shapiro, 1991). This agrees with the findings that all major vertebrate CK isoenzymes are able to interact, although to different extents, with model membranes (Rojo *et al.*, 1991a).

Energy utilization by the flagellum of sea urchin sperm is tightly coupled to the rate of energy production by the mitochondria. This coupling was shown to be mediated by a PCr shuttle since (i)  $^{31}\text{P}$ -n.m.r. results confirmed that in sperm, PCr is used as an acute source of energy (Christen *et al.*, 1983), and (ii) inhibition of sperm CK with increasing, but low, concentrations of dinitrofluorobenzene led to a progressive flagellar wave attenuation starting at the distal end of the sperm tail (Tombs *et al.*, 1987), though mitochondrial respiration was not affected (Tombs & Shapiro, 1985). Tail movement, however, was restored when these CK-inhibited spermatozoa were demembrated and transferred into a medium containing MgATP (Tombs *et al.*, 1987). These results provide strong support for the idea that the CK/PCr system is an obligatory link in the transport of high energy phosphates between the mitochondria near the sperm head and the axoneme running the length of the sperm tail, which depending on the species, may be as long as 100  $\mu\text{m}$ . Thus, in these highly polarized cells, the 'transport of energy' as ATP seems to be severely diffusion-limited and is facilitated instead by PCr. Despite appropriate controls performed by Shapiro and colleagues, some reservations concerning the 'absolute' specificity of dinitrofluorobenzene for CK may still be justified, even when the reagent is used at very low concentrations. Nevertheless, the sperm model is still the best example for the PCr circuit at work *in vivo*.

#### CK isoenzyme compartmentation and function in smooth muscle and intestinal epithelial cells

While the main CK isoform in smooth muscle is brain-type BB-CK, small amounts of MB- and traces of MM-CK were also found in this tissue (Iyengar *et al.*, 1982). Only recently was Mi-CK, mostly in its octameric form, detected in mitochondrial fractions of intestinal and vascular smooth muscles of guinea pig (Ishida *et al.*, 1991) and rat (Payne *et al.*, 1991). Whereas chicken small intestine also contained significant amounts of Mi-CK (our unpublished work), in chicken gizzard, which contains high levels of BB-CK (Quest *et al.*, 1990a), no Mi-CK has been found so far (Schlegel *et al.*, 1988a; Ishida *et al.*, 1991). Since PCr levels are significantly lower in smooth compared to striated muscle (Iyengar, 1984; Hellstrand & Vogel, 1985), e.g. 1–3 mM versus 20–30 mM, respectively, the kinetic characteristics of smooth muscle B-CK, namely, the lower  $K_m$  values for ADP and PCr (Iyengar *et al.*, 1982; Quest *et al.*, 1990b), are particularly suited for the role of CK in maintaining a constant level of ATP at the expense of the available cellular PCr. Furthermore, for B-CK from chicken and mouse, it was shown that the  $K_m$  for PCr is regulated by phosphorylation of the enzyme (Quest *et al.*, 1990b; Chida *et al.*, 1990a,b).

The presence of Mi-CK in mitochondria of guinea-pig smooth muscles may provide the molecular basis for the dependence of PCr production on oxidative metabolism, i.e. in the guinea-pig taenia caeci smooth muscle, which responds to stimulation with very long tonic contractures, some 60% of the total ATP demand is met by oxidative phosphorylation (Ishida & Paul, 1990). This was corroborated by  $^{31}\text{P}$ -n.m.r. studies using porcine arteries, where a two-site molecular exchange between ATP and PCr due to CK reactions was assumed (Clark & Dillon, 1990).

Thus, one may postulate that these sites in a smooth muscle cell correspond to cytosolic BB-CK (possibly associated at the subcellular level with sites of high ATP turnover) and to Mi-CK (working in the opposite direction within the mitochondria), forming a PCr circuit also in mammalian smooth muscles (Ishida *et al.*, 1991). In contrast, smooth muscle strips from chicken gizzard respond to stimulation with relatively fast phasic contractions without a tonic component (Fischer & Pfitzer, 1989). Thus, the presence of Mi-CK in guinea-pig smooth muscles, but not in chicken gizzard, reflects the different physiological properties of these muscles (Ishida *et al.*, 1991).

In the uterus, PCr levels increase significantly before labour, when large metabolic demands are made on the contracting myometrium; they remain high during parturition and return to pre-partum levels within a week after birth (Dawson & Wray, 1983), thus indicating that PCr is also critically involved in the energetics of uterine contraction. Interestingly, in uterus, B-CK gene transcription is oestrogen-induced (Reiss & Kaye, 1981).

High concentrations of BB-CK were detected in smooth muscle and in epithelial cells of the human colon (Jockers-Wretou *et al.*, 1977). In intestinal epithelial cells, the discrete subcellular compartmentation of BB-CK, concentrated distinctly in the brush border terminal web region and partially associated with the brush border itself, and of Mi-CK, located in the mitochondria just subjacent to the terminal web, were shown to be of physiological relevance by *in vitro* contraction experiments (Gordon & Keller, 1991). When in these polarized cells, high energy phosphate was supplied as PCr, brush border BB-CK imparted to the circumferential-ring myosin a selective accessibility of PCr and thus of ATP over other ATPases, suggesting a specific functional coupling of BB-CK with brush border myosin.

#### Membrane association and function of CK in electrocytes of electric fish

High concentrations of CK are also present in the electric organ of electric fish (Barrantes *et al.*, 1983a). The so-called non-receptor, peripheral  $\nu_2$ -protein, found in highly purified preparations of acetylcholine receptor-rich (AChR-rich) membranes obtained from *Torpedo marmorata* electric organ, was identified as membrane-associated CK (Barrantes *et al.*, 1983b). Biochemical and immunohistochemical studies showed that, as in muscle, the majority of CK in electrocytes is soluble (Barrantes *et al.*, 1985), but significant fractions of the enzyme were also localized *in situ* at the post-synaptic AChR-rich membrane, on synaptic vesicles at the innervated ventral side of electrocytes, and on isolated AChR-rich vesicles (Wallimann *et al.*, 1985; Gysin *et al.*, 1986). Most importantly, additional CK was also found in association with the entire canicular membrane system at the non-innervated dorsal face of the same cells (Wallimann *et al.*, 1985) where the ATP-requiring  $\text{Na}^+/\text{K}^+$ -ATPase is highly concentrated.

Some conflicting reports on the CK isoenzymes present in electrocytes have appeared in the literature. While some groups claimed that in *Torpedo* electrocytes, only the muscle-type MM-CK is synthesized (Witzemann, 1985), others demonstrated the presence of at least two CK isoforms in these cells (Barrantes *et al.*, 1985; Gysin *et al.*, 1986). Based on the much higher immunoreactivity with rabbit anti-chicken B-CK relative to anti-M-CK antibodies, Barrantes *et al.* (1983a,b, 1985) concluded that the  $\nu_2$ -protein corresponds to a brain-type B-CK isoenzyme. In contrast, Gysin *et al.* (1986), using homologous anti-(*Torpedo* CK) antibodies, presented conclusive evidence that the major CK species in electrocytes is muscle-type M-CK (see also Perryman *et al.*, 1985), that significant amounts of a CK isoform, tentatively identified as MB-CK, are also present, and that the enzyme bound to highly purified AChR-rich membranes is

muscle-type CK. Two CKs were cloned from *Torpedo californica* and *T. marmorata* electric organ (West *et al.*, 1984; Giraudat *et al.*, 1984). Interestingly, sequence comparison with avian and mammalian CK isoenzymes showed that neither of the *Torpedo* CK sequences fell definitely into either the muscle or brain class, but instead were both sharing M- and B-like CK domains (Babbitt *et al.*, 1986), thus explaining the apparently aberrant cross-reactivity of heterologous anti-B-CK antibodies. Therefore, the categorization of CK into brain or muscle isoenzymes may not be valid for species divergent from the mammalian or avian system from which such categorization arose. The correct assignment of these CK isoenzymes in electric fish has distracted the attention away from the physiological implications of CK at these various subcellular locations, as proposed by Wallimann *et al.* (1985).

In electric organs of various electric fish, the levels of CK and PCr are high, although aerobic and anaerobic metabolic synthetic pathways are slow (Blum *et al.*, 1990). Upon discharge of the electric organ, [PCr] rapidly falls (Borroni, 1984) due to activation of the  $\text{Na}^+/\text{K}^+$ -ATPase which is the major energy-utilizing pathway in electrocytes (Blum *et al.*, 1990). This is supported by the fact that ouabain, a specific inhibitor of the  $\text{Na}^+/\text{K}^+$ -ATPase, prevents PCr depletion (Blum *et al.*, 1991). By saturation transfer  $^{31}\text{P}$ -n.m.r., a coupled activity of CK and the sodium pump, both enzymes being membrane-bound and co-localized in the dorsal electrocyte membrane (Wallimann *et al.*, 1985), was directly demonstrated *in vivo* (Blum *et al.*, 1991). Thus, in electrocytes as in muscle, high energy demands, caused by electric discharge and subsequent recharge, are met at the expense of PCr, and recovery of the electric organ is closely related to the restoration of PCr levels (Borroni, 1984). In addition, the release of acetylcholine from *Torpedo* synaptosomes, where CK was also localized (Wallimann *et al.*, 1985), was shown to be severely affected by inhibition of CK by dinitrofluorobenzene (Dunant *et al.*, 1988). Since in electrocytes, the ATP synthetic pathways are slow, but upon discharge, large amounts of energy are needed at once, these cells represent an instructive example for an almost exclusive 'energy buffer function' of the CK/PCr system.

#### Cellular and subcellular compartmentation of CK isoenzymes in retina and photoreceptor cells

High amounts of CK activity were found in retina where two CK isoenzymes, BB-CK and Mi-CK, are present in several layers and cell types (Wallimann *et al.*, 1986a). In adult retina, most of the CK is concentrated within the photoreceptor cell layer, as judged from immunofluorescence staining. Other neuronal and glial cells stained to a lesser extent. In photoreceptors, BB-CK was found in the myoid and ellipsoid portions of the inner segments, as well as in the peripheral region of the outer segments (ROS), partially in association with the ROS plasma membrane (Hemmer *et al.*, 1989; Wegmann *et al.*, 1991a). Mi-CK is specifically sequestered in the ellipsoid portion of the inner segment where most of the photoreceptor's mitochondria are clustered (Wallimann *et al.*, 1986a). Mi-CK has been localized in these mitochondria along the cristae membranes as well as at those sites where outer and inner membrane are in close proximity, presumably at the contact sites (Wegmann *et al.*, 1991a). Interestingly, Mi-CK is expressed in retina rather late during embryonic development of the chicken and turned out to be a good marker of photoreceptor differentiation.

Photoreceptor cells, like spermatozoa, are highly polarized. ATP synthesis, that is, glycolysis as well as oxidative phosphorylation, is confined entirely to the inner segment (Lowry *et al.*, 1961; Berger *et al.*, 1980; Wallimann *et al.*, 1986a; Hemmer *et al.*, 1989), whereas many energy-requiring reactions take place in the outer segment, e.g. regeneration of GTP, cyclic GMP and

ATP, utilized during phototransduction (see Chabre & Deterre, 1989). The sites of ATP synthesis and utilization are therefore spatially separated by appreciable distances. The rather narrow connecting cilium between the inner and outer segment may additionally reduce the diffusion flux. Thus, the presence of segregated CK isoenzymes in photoreceptor cells, which are subject to high and fluctuating energy requirements, as well as their relatively high content of PCr (Wallimann *et al.*, 1986a) are indicative for a PCr circuit involved also in the bioenergetics of vision.

Besides photoreceptor cells and neuronal cells of the retina, Müller glia cells also contain appreciable amounts of B-CK as judged from immunofluorescence staining (Wegmann *et al.*, 1991a), indicating that these glia cells possess a high energy status and that the CK/PCr system may be involved in the energetics of K<sup>+</sup>-resorption and other ATP-dependent functions attributed to glia cells (Newman, 1985).

### CK in brain

Aerobic glycolysis is the primary pathway of ATP synthesis in brain (Sokoloff, 1989). With only small stores of glucose, glycogen and O<sub>2</sub>, the rate of glycolysis in brain is closely coupled to cerebral blood flow and oxygen uptake. These rates in turn are coupled to the utilization of ATP by factors such as [ADP], local pH, or extracellular [K<sup>+</sup>] (Holtzman & Olson, 1983). A rapidly available source for ATP synthesis in brain is the CK/PCr system. The high activity of CK in brain (Norwood *et al.*, 1983) justifies the assumption that CK is a key enzyme in the energy metabolism of this tissue.

**CK isoenzymes in brain.** BB-CK is the major 'cytosolic' CK isoenzyme present in brain (Eppenberger *et al.*, 1967; Quest *et al.*, 1989). For chicken B-CK, a considerable heterogeneity was found, with two major B-type subunits and additional subspecies arising from alternative ribosomal initiation (Soldati *et al.*, 1990) and post-translational modifications (Quest *et al.*, 1990b, 1991). In adult human brain, the presence of MM-CK has also been reported (Lindsey & Diamond, 1978; Hamburg *et al.*, 1990). While Northern blot analysis failed to detect any M-CK message, this determination was only achieved by polymerase chain reaction (Hamburg *et al.*, 1990). Initial immunolocalization studies suggest that MM-CK is restricted to specific regions of the brain (Hamburg *et al.*, 1990).

Jacobs *et al.* (1964) and Swanson (1967) reported on CK activity associated with brain mitochondria, which was later identified and characterized as authentic brain Mi-CK (Booth & Clark, 1978; Wevers *et al.*, 1981; Wyss *et al.*, 1990). Recently, Mi<sub>a</sub>-CK from brain, and Mi<sub>b</sub>-CK from heart were shown to be two different isoenzymes (Schlegel *et al.*, 1988b; Hossle *et al.*, 1988; Wyss *et al.*, 1990; Haas *et al.*, 1989; Haas & Strauss, 1990; Payne *et al.*, 1991), but both form dimeric and octameric molecules (Schlegel *et al.*, 1988b; Wyss *et al.*, 1990). Furthermore, Mi<sub>a</sub>-CK was found to be localized preferentially as octamers in mitochondrial boundary membrane contact site fractions of brain mitochondria (Kottke *et al.*, 1988). Thus, the major enzymes in brain and neural tissues are BB-CK and Mi<sub>a</sub>-CK, but the presence of small amounts of MM-CK has now also to be considered.

**Regional and cellular compartmentation.** CK is an abundant enzyme in the entire brain, but microdissection studies already pointed to the existence of regional differences in CK content and PCr concentrations, with CK activity varying more than two-fold among different areas of the brain (Chandler *et al.*, 1988). In cerebellar cortex, especially in the molecular layer, higher levels of CK and PCr were measured than in white matter (Maker *et al.*, 1973). The cerebellum, striatum and pyramidal tracts also contain higher CK activities than whole brain (Manos

*et al.*, 1991). These findings are in accordance with recent <sup>31</sup>P-n.m.r. imaging showing that the flux through the CK reaction as well as [PCr] are significantly higher in grey compared to white matter (Cadoux-Hudson *et al.*, 1989). In addition, creatine was also found by magnetic resonance imaging to be unevenly distributed in the brain (Moonen *et al.*, 1990).

Early localization studies in the cerebellum by histochemical staining for CK activity displayed a more intense staining in the molecular layer than in the granular layer of the cerebellar cortex and much less staining in the cerebellar white matter (Khan *et al.*, 1971), which is in agreement with the above findings. The CK activity was especially high in cerebellar Purkinje cells (Khan, 1976; Kato *et al.*, 1986). Using a rabbit anti-rabbit M-CK auto-antibody, Ikeda & Tomonaga (1987) found by immunoperoxidase staining of mouse brain a distinctive group of positive neurons in the zona incerta and the lateral hypothalamic area, as well as positive staining of Purkinje cells and their dendrites. In addition, positive immunostaining for CK was also demonstrated in nerve terminals in the hypothalamic area and the superior colliculus (Ikeda & Tomonaga, 1988). CK, however, is not restricted to neuronal cells, but was also found in oligodendrocytes (Manos *et al.*, 1991), astrocytes (Thompson *et al.*, 1980; Yoshimine *et al.*, 1983; Manos *et al.*, 1991) and cerebellar Bergmann glia cells (Scalabrini *et al.*, 1989). Surprisingly, the levels of CK seem to be higher in certain glia cells than in neurons (Scalabrini *et al.*, 1989), a fact which has been confirmed with cultured, purified brain cells (Manos *et al.*, 1991), indicating that oligodendrocytes contain nearly fourfold higher levels of CK activity than do neurons (Manos *et al.*, 1991). Thus, these authors speculated that in oligodendrocytes, representing cells with large energy requirements, CK function is coupled to myelin synthesis, transport or assembly. This notion is supported by the fact that CK levels continue to rise during development within the period of most active myelination (see Manos *et al.*, 1991). Glia cells contain not only relatively high amounts of CK and PCr, but, according to recent findings with cultured astroglia, also use an active, saturable Na<sup>+</sup>-dependent creatine uptake system which is competitively inhibited by β-guanidinopropionic acid (GPA), whereas a similar transport system was not observed in neuron-rich cell cultures (Möller & Hamprecht, 1989). The high energy status of glia cells is surprising, because initially, these cells were thought to fulfil only a supportive function for neuronal cells, e.g. to serve as developmental scaffolds. However, it is fully in line with recent evidence that glia cells play an important role in a number of ATP-dependent processes, e.g. resorption of K<sup>+</sup> and neurotransmitters released from neurons, and maintenance of ion balance at the blood-brain barrier (Newman, 1985; for review see Kimelberg & Norenberg, 1989).

**Subcellular compartmentation of CK in brain.** Unlike in muscle, sperm and electrocytes, little is known about the subcellular distribution and specific function of CK in brain. This may in part be due to the very high degree of regional and cellular complexity of the vertebrate brain. While Mi<sub>a</sub>-CK is clearly restricted to mitochondria, only little evidence for specific subcellular association of 'soluble' brain CK is available at this time. Similar to electrocytes (Wallimann *et al.*, 1985), brain CK has been found in association with synaptic vesicle (Friedhoff & Lerner, 1977) and plasma membranes (Lim *et al.*, 1983). As shown for electrocytes, brain CK, bound to synaptic vesicles and to the plasma membrane, is also likely to be involved in acetylcholine release (Dunant *et al.*, 1988) and, in conjunction with the Na<sup>+</sup>/K<sup>+</sup>-ATPase, in the maintenance of membrane potentials (Blum *et al.*, 1991).

Furthermore, CK, together with nerve-specific enolase, belongs to a group of proteins known as slow component b (SCb). These proteins are synthesized in neuronal cell bodies and

are directed by axonal transport to the axonal extremities (Brady & Lasek, 1981; Oblinger *et al.*, 1987). The question of whether CK participates in the actual energetics of axonal transport remains to be answered. However, the association of a fraction of 'soluble' CK with SCb proteins shows an intracellular compartmentation of the enzyme also in nerve cells. The fact that during preparation of neuron-specific enolase, brain CK co-chromatographs with the latter glycolytic enzyme (Gerbitz *et al.*, 1983), may also indicate a functional coupling of brain CK with glycolysis, as was demonstrated in muscle (see above).

**Developmental changes in CK activity and phosphorus metabolites.** In the altricial neonate, including mouse, rat, rabbit, pig and human, marked quantitative and qualitative changes in the physiology of ATP metabolism occur postnatally (see Holtzman *et al.*, 1991). Similarly, rather dramatic postnatal increases in total CK activity (Booth & Clark, 1978) as well as in PCr content were noted (Tofts & Wray, 1985; Norwood *et al.*, 1983). For example, in the narrow time-window between days 12–15 of postnatal development of mouse and rat, (i) the *in vivo* rate of CK-catalysed ATP synthesis increased 4-fold, as measured by saturation transfer  $^{31}\text{P}$ -n.m.r. (Holtzman *et al.*, 1991), (ii) the brain developed the capacity to increase ATP synthesis in response to sudden changes in energy demand, and (iii) a population of cerebral brain mitochondria appeared with tight contacts between inner and outer membranes (Holtzman *et al.*, 1979). These observations suggest that the increase in CK reaction rates is due to the appearance of Mi-CK, an interpretation supported by measurements *in vitro* (Booth & Clark, 1978; Norwood *et al.*, 1983). Accordingly, in immature brain, hypoxia resulted in an almost complete loss of PCr and an about 50% decrease in [ATP], whereas in adult brain, [ATP] remained rather stable, with PCr levels decreasing by 30% (see Holtzman *et al.*, 1991; D. Holtzman, personal communication). Interesting with respect to brain development is the acute stimulation of total brain CK activity by vitamin D metabolites observed in the developing cerebellum (Binderman *et al.*, 1988).

**Brain CK and PCr in mice fed an analogue of creatine.** In skeletal muscle, feeding of mice with GPA results in an almost complete replacement of PCr by the phosphorylated analogue GPAP (Shoubridge & Radda, 1984). In brain, a GPA-inaccessible pool of PCr which could not be replaced by the analogue was found by  $^{31}\text{P}$ -n.m.r. (Holtzman *et al.*, 1989). This residual fraction of PCr was hypothesized to represent the same compartmentalized pool which is stable in hypoxic or seizing animals. This view is consistent with the observation that cellular and subcellular compartmentation of energy metabolism exists in brain, e.g., higher rates of aerobic glycolysis and the capacity to increase this rate with increased energy demand are characteristic of astrocytes rather than of neurons (Holtzman & Olson, 1983; see Holtzman *et al.*, 1989). These data indicate that the control, coupling and kinetics of the PCr/CK/ATP system in brain are much more complex *in vivo* than previously appreciated from studies *in vitro* (Siesjö, 1978) and that further experimentation is needed to understand these complexities which, nevertheless, may be relevant to clinical conditions, such as the pathogenesis of hypoxia in the neonate, or of seizures and stroke.

#### CK in kidney

Cellular compartmentation of B-CK, mainly confined to the epithelial cells of the thick ascending limb of the Henle's loop and the collecting tubule of the renal medulla, as well as to certain epithelial cells in cortical tubules, has been described (Ikeda, 1988). The defined cellular localization of B-CK at these places raises the possibility that CK may participate in certain renal tubular functions, e.g. by supplying ATP to the membrane-

bound ion exchangers and ion pumps present in these subpopulations of cells. Although Mi-CK is expressed at high concentrations also in kidney (Payne *et al.*, 1991), the cellular and subcellular localization of this isoenzyme is still unknown and thus, the question of whether a PCr circuit is also operational in certain kidney cells remains to be answered.

#### REGULATION OF CK AND CONNECTION OF THE ENZYME TO SIGNALLING PATHWAYS

Since intracellular ATP/ADP ratios regulate a variety of cellular processes and since the CK system is involved in regulation of local ATP/ADP ratios (Watts, 1971; Newsholme *et al.*, 1978; Rossi *et al.*, 1990), it is of interest to consider that CK itself may also be subject of regulation, e.g. by post-translational modification. Indeed, BB-CK was shown to be a phospho-protein (Mahadevan *et al.*, 1984; Quest *et al.*, 1990b). Phosphorylation decreases the  $K_m$  of chicken and mouse BB-CK for PCr by approximately a factor of two, e.g. from 1.6 mM to 0.8 mM-PCr for the chicken enzyme (Quest *et al.*, 1990b; Chida *et al.*, 1990a). This change is especially relevant in brain where the concentration of PCr is only 4–6 mM (Cadoux-Hudson *et al.*, 1989), where the PCr concentration rapidly falls during hypoxia or seizures (Thulborn *et al.*, 1982) and where evidence for compartmentation of PCr, as in muscle, has also been reported (Holtzman *et al.*, 1989). By stimulation of keratinocytes with phorbol esters, protein kinase C (PKC) was identified as a likely candidate to be responsible for CK phosphorylation *in vivo* (Chida *et al.*, 1990b). In addition, covalent modification of CK by autophosphorylation has recently been discovered (Hemmer *et al.*, 1991).

Thus, CK isoenzymes are subject to complex regulation not only as far as their association with subcellular structures is concerned (see above), but also with respect to enzyme kinetics, since the CK system seems to be connected to signal transduction pathways via PKC (Mahadevan *et al.*, 1984; Quest *et al.*, 1990b; Chida *et al.*, 1990b; see also Wallimann *et al.*, 1989). It is conceivable that phosphorylation-dependent membrane attachment and detachment as a mechanism of reversible membrane targeting could also take place for the CK isoenzymes, as has very recently been shown for the myristoylated alanine-rich PKC substrate (Thelen *et al.*, 1991). Furthermore, it is likely that the enzyme is connected to signal transduction pathways also at a different level, that is, by locally providing ATP for protein kinases, some of which, like PKC, are in part also compartmentalized themselves.

#### CONCLUDING REMARKS AND PERSPECTIVES

The tissue-specific distribution, subcellular localization and function of CK isoenzymes in tissues and cells with high and fluctuating energy requirements, as well as the molecular structure of Mi-CK, point to an important physiological role of the highly compartmentalized CK system for cellular energetics.

The phosphocreatine circuit (PCr circuit) proposed here serves (i) as an 'energy buffering system' and (ii) as a regulated 'energy transport and distribution network' connecting intracellular sites of high energy demand with sites of ATP production. At these sites, CK isoenzymes are specifically localized, and the 'transport of energy' between them is accomplished by PCr and Cr. CK, representing a low-threshold sensor of ADP, further serves by its enzymic reaction (iii) to prevent, upon cell activation, a rise of [free ADP] which would otherwise inhibit ATP-dependent processes and, in addition, lead to a net loss of cellular adenine nucleotide pools by the action of adenylate kinase, AMP-deaminase and 5'-nucleotidase. Furthermore, the CK/PCr system serves (iv) to prevent a local rise in  $[\text{H}^+]$  and thus also a

global acidification of the cell, caused by high ATP breakdown. This is achieved by the functional coupling of the CK reaction to various cellular ATPases. Additionally, since the net breakdown of PCr in conjunction with ATPase reactions leads to a release of  $P_i$ , the CK reaction stimulates indirectly also glycogenolysis. Furthermore, the CK/PCr circuit system serves (v) as a 'regulatory system' for the maintenance of appropriate subcellular ATP/ADP ratios and, by keeping this ratio high in the vicinity of cellular ATPases, increases the thermodynamic efficiency of ATP hydrolysis which itself is a crucial parameter in energy homeostasis. The correspondingly high free energy for the resynthesis of ATP is obtained on the mitochondrial side of the PCr circuit, where the Mi-CK is part of a multi-enzyme compartment for 'metabolic channeling', and where the inner mitochondrial membrane potential acts on the free energy of hydrolysis of ATP in two steps: during the synthesis and during the electrogenic export of ATP in exchange for ADP.

Mi-CK, located on the outer side of the inner mitochondrial membrane as well as at the inner/outer membrane contact sites, serves to facilitate, by functional coupling with the ATP/ADP translocator (ANT), (i) the electrogenic export of mitochondrial matrix-generated ATP in exchange for ADP through the inner mitochondrial membrane, (ii) the transphosphorylation of this ATP into PCr, and by doing so, (iii) conserves the free energy of hydrolysis of ATP just exported by the ANT. In addition, by being functionally coupled to the outer mitochondrial membrane pore, Mi-CK is probably also involved in (iv) the regulation of the import of Cr into mitochondria and of the export of PCr into the cytosol. Therefore, through the concerted kinetic coupling of the three enzymes ANT, Mi-CK and mitochondrial pore protein (porin), a microcompartment important for mitochondrial 'energy transport' and cellular energy metabolism is formed (see the model in Fig. 3).

The CK/PCr circuit seems to fulfil the requirements of a highly organized 'energy buffer' and 'energy transport' system, as well as that of a 'regulatory system' for the control of subcellular ATP/ADP ratios, together leading to a more efficient utilization of energy in thermodynamic terms. Depending on the metabolic needs of a cell or tissue, one of these different functions of the CK/PCr circuit may be dominating, e.g. in fast-twitch, glycolytic muscle fibres, the 'buffer function' may be more prominent than the 'transport function', whereas in slow-twitch, oxidative or cardiac muscle, as well as in spermatozoa or photoreceptor cells, the 'transport function' may be of greater relevance than the 'buffer function' (Figs. 2 and 3).

Future studies on the CK system should focus on the isolation and functional characterization of further CK/ATPase micro-compartments from different tissues, using a multidisciplinary biochemical and immunohistochemical approach which has been quite successfully applied to M-line- and SR-bound CK (Wallimann & Eppenberger, 1985; Rossi *et al.*, 1990).

In addition, the elucidation of the functional details of Mi-CK in mitochondrial energy transfer requires more experimentation. The interaction of Mi-CK directly with inner and outer mitochondrial membranes (Rojo *et al.*, 1991a,b) and with proteins thereof deserves full attention in the future. Furthermore, solving the crystal structure of CK, especially that of the Mi-CK octamer (Schnyder *et al.*, 1990), is a project of high priority, as the detailed knowledge of the structure of this 'energy channelling molecule' should also provide valuable information about its function within the mitochondrion. These biochemical, cell biological and molecular biological approaches will be complemented by non-invasive  $^{31}\text{P}$ -n.m.r. studies applied to isolated organelles (mitochondria), tissues and whole animals with improved resolution in space and time.

The possibility to over-express cloned CK genes coding for B-

CK, M-CK and Mi-CK isoenzymes in *Escherichia coli* (Koretsky & Traxler, 1989; Babbitt *et al.*, 1990; Chen *et al.*, 1991; Furter & Wallimann, 1991, respectively), in yeast (Brindle *et al.*, 1990) or in transgenic mice (Koretsky *et al.*, 1990) offers a variety of advantages, e.g. for obtaining large amounts of homogeneous enzyme for kinetic and structural studies. Very specific questions can also be asked in the future by applying site-directed mutagenesis to study CK isoenzyme structure and function. *In vivo*  $^{31}\text{P}$ -n.m.r. experiments with cells that otherwise would not express CK will be useful to address questions concerning the determination of [free ADP] and of ATP/ADP ratios in the cell (Koretsky *et al.*, 1990). Most interestingly, in experiments with transgenic mice expressing variable amounts of BB-CK in liver, metabolic questions concerning, for example, the role of ADP during a fructose load can be approached (Brosnan *et al.*, 1991).

The data gathered by such powerful multidisciplinary approaches should provide further insights into the structural/functional aspects of CK and the physiological function of the CK/PCr system. This information may provide important clues for muscle disease and pathology. Relevant in this respect is the recent finding that the highly ordered crystalline mitochondrial inclusions, particularly occurring in mitochondria of 'ragged-red' skeletal muscle fibres in patients suffering from chronic progressive external ophthalmoplegia or from other mitochondrial encephalomyopathies, are highly enriched in Mi-CK, representing the major component of these mitochondrial inclusions (Stadhouders *et al.*, 1990, 1991).

#### Note added in proof

Very recently the complete gene structure of the human sarcomeric and ubiquitous Mi-CK, the location of these genes on chromosomes 5 and 15 respectively and regulatory elements of the tissue-specific mitochondrial CK genes have been described (Klein *et al.*, 1991).

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