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III-2 Creatine kinase in non-muscle tissues and cells

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Abstract

Over the past years, a concept for creatine kinase function, the 'PCr-circuit' model, has evolved. Based on this concept, multiple functions for the CK/PCr-system have been proposed, such as an energy buffering function, regulatory functions, as well as an energy transport function, mostly based on studies with muscle. While the temporal energy buffering and metabolic regulatory roles of CK are widely accepted, the spatial buffering or energy transport function, that is, the shuttling of PCr and Cr between sites of energy utilization and energy demand, is still being debated. There is, however, much circumstantial evidence, that supports the latter role of CK including the distinct, isoenzyme-specific subcellular localization of CK isoenzymes, the isolation and characterization of functionally coupled *in vitro* microcompartments of CK with a variety of cellular ATPases, and the observed functional coupling of mitochondrial oxidative phosphorylation with mitochondrial CK. New insight concerning the functions of the CK/PCr-system has been gained from recent M-CK null-mutant transgenic mice and by the investigation of CK localization and function in certain highly specialized non-muscle tissues and cells, such as electrocytes, retina photoreceptor cells, brain cells, kidney, salt glands, myometrium, placenta, pancreas, thymus, thyroid, intestinal brush-border epithelial cells, endothelial cells, cartilage and bone cells, macrophages, blood platelets, tumor and cancer cells. Studies with electric organ, including *in vivo* ^{31}P-NMR, clearly reveal the buffer function of the CK/PCr-system in electrocytes and additionally corroborate a direct functional coupling of membrane-bound CK to the Na^+/K^+-ATPase. On the other hand, experiments with live sperm and recent *in vivo* ^{31}P-NMR measurements on brain provide convincing evidence for the transport function of the CK/PCr-system. We report on new findings concerning the isoenzyme-specific cellular localization and subcellular compartmentation of CK isoenzymes in photoreceptor cells, in glial and neuronal cells of the cerebellum and in spermatozoa. Finally, the regulation of CK expression by hormones is discussed, and new developments concerning a connection of CK with malignancy and cancer are illuminated. Most interesting in this respect is the observed upregulation of CK expression by adenoviral oncogenes. (Mol Cell Biochem **133/134:** 193–220,1994)

Key words: creatine kinase, functional coupling with cellular ATPases, spermatozoa, electrocytes, retina, cerebellum

Abbreviations: M-CK, B-CK and Mi-CK refer to muscle-type, brain-type and mitochondrial-type creatine kinase, respectively, with the cytosolic isoforms MM-, MB- and BB-CK forming dimers and Mi-CK forming dimers as well as octamers; BGC – Bergmann glial cell; GL – granule cell layer; ML – molecular layer; PN – Purkinje neuron; PCr – phosphoryl-creatine; Cr – creatine; cCr – cyclo-creatine; PcCr – phosphoryl-cyclo-creatine; ROS – photoreceptor rod outer segment; IS – inner segment; DNFB – 2,4-dinitro-fluoro-benzene; PEP – phosphoenolpyruvate; PK – pyruvate kinase; GPA – 3-guanidino-propionic acid

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Introduction

The creatine kinase/phosphoryl-creatine circuit hypothesis for energy homeostasis in muscle and other cells with intermittently high and fluctuating energy requirements

Cells require energy to survive and to carry out the multitude of tasks that characterize biological activity. Cellular energy demand and supply are generally balanced, and tightly regulated for economy and efficiency of energy use. The enzyme creatine kinase (CK; ATP: creatine N-phosphoryl-transferase, EC 2.7.3.2) omit, plays a key role in the energy metabolism of cells with intermittently high and fluctuating energy requirements, such as skeletal and cardiac muscle, neural tissues like brain and retina, or spermatozoa and electrocytes. It catalyzes the reversible transfer of the phosphoryl group from phosphorylcreatine (PCr) to ADP, to regenerate ATP. The enzyme is found in two cytosolic isoforms ('ubiquitous' B-CK and 'sarcomeric' M-CK) [1] and two mitochondrial isoforms (ubiquitous Mi_a-CK, and sarcomeric Mi_b-CK) [2–4].

Using biochemical fractionation and *in situ* immunolocalization techniques on skeletal and cardiac muscle tissue, one was able to show that in sarcomeric muscle, the CK isoenzymes, earlier considered as strictly soluble enzymes (see textbooks), are in fact not distributed evenly within the cells of these tissues [5, 6]. Instead, CK isoenzymes were found to be compartmentalized subcellularly in an isoenzyme-specific fashion [7]. The highly ordered structural organization of muscle and the prevalence in this tissue of specialized cellular ATPases, such as the acto-myosin ATPase and the SR-Ca^{2+}-ATPase, made it possible to study, by biochemical and immunohistochemical methods, the specific subcellular associations of CK with these muscular ATPases. So-called functionally coupled microcompartments could be identified and isolated *in vitro* (for recent reviews see [4] and [8] and refs. therein). For example, in sarcomeric muscle, some cytosolic M-CK is localized at the M-band [6], the sarcoplasmic reticulum (SR) and the plasma membrane. At these sites, M-CK is functionally coupled to the myofibrillar acto-myosin ATPase [9–11], the SR Ca^{2+}-ATPase [12, 13] and at the plasma membrane Na^+/K^+ ATPase [14], respectively, and utilizes PCr for *in situ* regeneration of ATP (Fig. 1). The presence of CK at these sites of high energy demand and the formation of functionally coupled microcompartments conveys kinetic and thermodynamic advantages to the system [8, 15]. It was demonstrated that the myofibrillar actin-activated myosin ATPase and the SR Ca^{2+}-ATPase in isolated microcompartments have priviledged access to ATP generated by bound CK, even in the presence of exogenously added ATP-supplying systems or exogenous ATP traps [9–11, 16]. Interestingly, in an *in vitro* model system, using co-immobilized CK and myosin S1, a very similar functional coupling via channelling of substrates and products between the two enzymes in an unstirred layer was observed [17].

The cytosolic as well as subcellularly associated CKs, together with the mitochondrial CK isoforms are thought to constitute an intricate cellular energy buffering and transport system interconnecting via PCr and creatine (Cr) omit, intracellular sites of high-energy phosphate production, i.e. glycolysis and oxidative phosphorylation, with sites of energy consumption, e.g. myofibrils and membrane ion pumps [8] (see Fig. 1). Such a tightly regulated communication between mitochondrial and 'cytosolic' CK isoforms has mainly been demonstrated for muscle, the tissue of choice for studying bioenergetics.

The mitochondrial CK isoenzyme [18, 19], Mi-CK, is located in the mitochondrial intermembrane space [20], where it is found along the entire inner membrane, but also at peripheral sites where inner and outer membranes are in close proximity [20, 21] (for reviews see [4, 8]). There, Mi-CK can directly transphosphorylate intramitochondrially produced ATP into PCr [22], which then is exported into the cytosol where it serves at relatively high concentration (5–40 mM, depending on the tissue) as an easily diffusible energy storage and transport metabolite (Fig. 1). Mi-CK, in contrast to the dimeric cytosolic CK isoenzymes, forms highly symmetrical, cube-like octameric structures that are characterized by a central channel running in parallel to the four-fold axis through the entire molecule [23, 24]. These Mi-CK octamers have the specific ability to peripherally bind to lipid membranes and, most importantly, to mediate contact site formation between inner and outer mitochondrial membranes *in vitro* [25]. Studies done with isolated mitochondria or permeabilized cell culture models, have shown functional coupling of mitochondrial CK with oxidative phosphorylation [22, 26–29]. This functional coupling of Mi-CK to oxidative phosphorylation occurs via the adenine nucleotide translocator (ANT) [22, 27, 29] which catalyzes the antiport of ATP versus ADP through the inner membrane. CK substrates and products also have to pass the outer mitochondrial

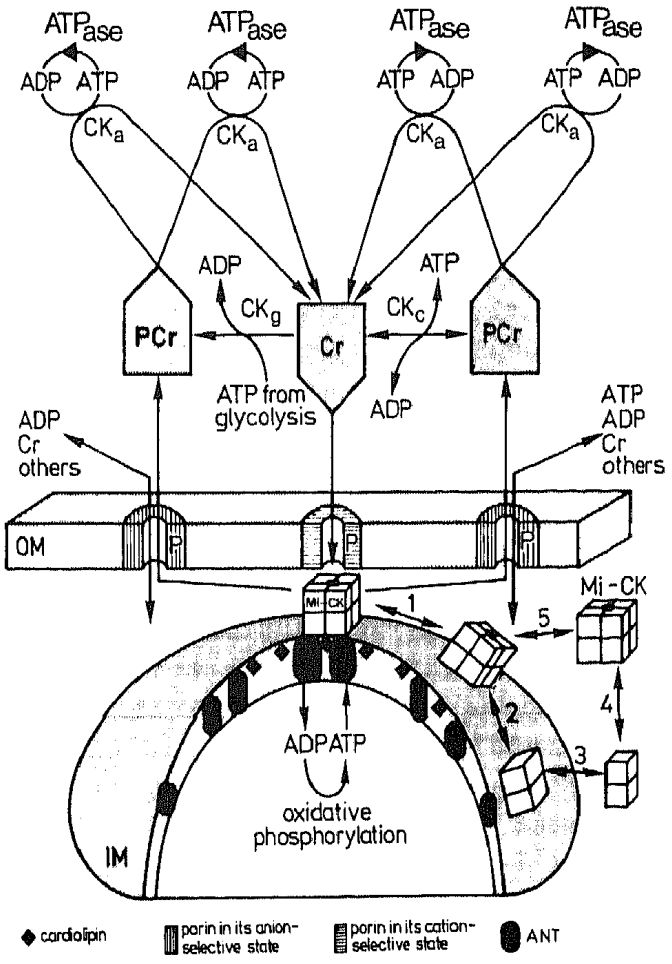

Fig. 1. The phospho-creatine circuit model for specialized cells with high and fluctuating energy metabolism. In a cell, ATP may be derived from two major synthetic pathways, that is, from oxidative phosphorylation (indicated at the bottom) and from glycogenolysis or glycolysis (GL, shown in the left middle part). Four major compartments of CK are indicated: i) strictly soluble cytosolic CK (CK_c) freely equilibrating PCr/Cr and ATP/ADP ratios in the cytosol (shown in the right middle part); ii) 'cytosolic' CK (CK_g) functionally coupled to glycolysis on the producing side of the PCr-circuit (shown in the left middle part), iii) 'cytosolic' CK (CK_a), specifically associated with subcellular structures at sites of high and fluctuating ATP requirements on the receiving end of the PCr-circuit, e.g. at the myofibrils (Refs. [9–11, 16], the sarcoplasmic reticulum (Refs. [12, 13]) and the sarcolemma (Ref. [14]), where functionally coupled CK microcompartments are formed with the myofibrillar acto-myosin ATPase, the Ca^{2+}-ATPase and the Na^+/K^+-ATPase, respectively (ATPase, at the top) [see text]; and finally, iv) mitochondrial CK (Mi-CK) being functionally coupled to oxidative phosphorylation (indicated at the bottom) (Refs. [22, 27, 30]). Note that in resting muscle, for example, the relative pool sizes of phosphoryl-creatine ([PCr] = ca. 20–40 mM) and creatine [Cr = ca. 5–15 mM] are much larger than those of the adenine nucleotides ([ATP] = ca. 3–5 mM; [ADP] = ca. 10–20 μM). Also note that PCr and Cr are smaller and less charged molecules compared to the adenine nucleotides (for Refs. see [8]). At the mitochondrial side, a cube-like Mi-CK octamer with an internal channel (Ref. [24]) is shown to interact with the inner (IM) as well as with the outer mitochondrial membrane (OM), thus stabilizing contacts between IM and OM (Ref. [25]). A Mi-CK octamer is depicted to interact with the ATP/ADP-translocator (ANT) of the IM, and with porin (P) of the OM, transiently forming a dynamic multienzyme 'channel' (Ref. [59]) at the so-called 'mitochondrial energy transfer contact sites' (Ref. [31]). The small black triangles in the IM and in association with ANT's represent cardiolipin molecules. ATP generated by oxidative phosphorylation, after transport through the IM by ANT in exchange for ADP, is transphosphorylated by Mi-CK to give PCr. Due to the functional coupling between ANT and CK (Refs. [22, 27]), PCr, as a net product of oxidative phosphorylation, leaves the mitochondrion through porin (P) of the OM in its high conductance, anion-selective state (for details see [4, 8, 31, 36]). Creatine (Cr), on the other hand, is entering at the contact sites into the intermembrane space, where the porin channel is thought to be in its cation-selective state due to capacitive coupling of the OM to the membrane potential of the IM, both being in close apposition at these sites (Ref. [31]). Possible regulatory aspects of Mi-CK in cellular energetics are depicted at the lower right, e.g. the reversible formation of contacts with ANT and P at the contact sites (arrows, number 1), the dynamic octamer/dimer equilibrium of the enzyme, while bound to the IM (arrows, number 2) or being in solution in the intermembrane space (arrows, number 4), as well as the differential pH-dependent association of the two oligomeric species of Mi-CK with the IM (3, for dimers; 5, for octamers), all observed *in vitro* (Refs. [23, 32]). According to this model, in a cellular system performing work, only small pools of adenine nucleotides (ATP and ADP) are turned over rapidly and in opposite direction at the producing (bottom and middle part of the figure) and the receiving end (top part of the figure) of the PCr-circuit. The model presented here stresses the functional coupling of ATP production with ATP utilization via CK and PCr, as well as the diffusional pathways of PCr and Cr. This pathway may be crucial for the cell at high work-load. However, parallel pathways involving a direct transport of ATP may also operate at the same time. This model, originally developed for muscle (Refs. [4, 8, 59]) is likely to be relevant also for sperm motility, photoreceptor cell function in the vertebrate retina, for smooth muscle, electrocytes, brush borders, etc., as well as for brain energetics (for reviews and references therein see Refs. [4, 8, 36, 38, 70, 71, 133, 140]).

membrane. Based on experiments showing that mitochondrial respiration can be effectively stimulated by extramitochondrial creatine [29, 30], leading to a net production of PCr by mitochondria, a functional coupling between Mi-CK and porin has also been postulated [4, 8, 31] (see Fig. 1).

The fact that Mi-CK is involved in the transphosphorylation, channelling and transport of energy-rich phosphates from mitochondria to the cytosol, together with the molecular structure of the Mi-CK octamer itself, which is very reminiscent of a 'channel protein', led to the hypothesis that Mi-CK could act as a connecting module between ANT and porin at the mitochondrial contact sites [4, 8, 31], thereby forming an efficient, tightly coupled multienzyme 'energy channel' [4, 8, 31] that combines the directed export of mitochondrial energy equivalents with the interconversion of matrix-generated ATP plus Cr into ADP plus PCr (see Fig. 1). Specific

features of Mi-CK, e.g. a dynamic octamer/dimer equilibrium which is influenced by physiological parameters [32], as well as the differential pH-dependent interaction of Mi-CK octamers and dimers with inner mitochondrial membranes observed *in vitro* [23, 32] (see Fig. 1) may be important parameters for the regulation of mitochondrial energetics. Based on these results, mainly obtained with muscle, the 'phospho-creatine shuttle' [16, 22, 33] or 'PCr-circuit' [8, 34] models have been formulated (see Fig. 1), and several main functions of the CK/PCr-system have been proposed (see [4, 8, 34–36].

First, the CK/PCr-system is thought to serve as a temporal energy buffer [37] keeping [ATP] and [ADP] steady and buffering [H^+]. This temporal buffer function prevents a rapid fall of [ATP] and a large build-up of [ADP] during cellular work and at the same time avoids an intracellular acidification due to the hydrolysis of ATP during work. These functions, which have also been proposed for CK in photoreceptor cell outer segments (ROS) [38], have recently been fully confirmed by transgenic animal approaches. For example, in mice expressing significant amounts of B-CK in liver, where normally no or very little CK is present, a strong buffering effect of CK on the ATP levels and the intracellular pH has been directly demonstrated upon perfusion of the liver under anoxic conditions [39–41]. In addition, also with the transgenic liver model it was shown that [ADP] is inversely proportional to the levels of B-CK expressed in these livers and that PCr protected ATP levels from the effects of a fructose load in the same animals [40]. Furthermore, the temporal buffer function of the CK system has very recently also been confirmed in muscle with gene-targeted null-mutant transgenic mice which do not express 'cytosolic' muscle-type MM-CK [42]. These mice display a distinct phenotype with significantly reduced muscle burst activity, that is, in the first phase of muscle contraction, normal peak tension is reached, but this peak tension rapidly falls to 50–60% of the control value seen with wild-type mice [42].

Second, the PCr-circuit serves to improve the thermodynamic efficiency of ATP hydrolysis by keeping intracellular [ADP] low and by maintaining ATP/ADP ratios high at those subcellular sites where CK is functionally coupled to ATP-requiring processes, like ion pumps (see CK_a, Fig. 1 top) that are largely dependent on a high affinity of ATP hydrolysis (A) in the sarcoplasm, with $A = \Delta G^{\circ}_{obs} - RT \ln ([ATP]/[ADP]*[Pi])$, where ΔG°_{obs} is the standard free energy of ATP hydrolysis, R is the gas constant and T is the absolute temperature [12, 13, 15, 43, 44]. Recent examples supporting this CK function are the observed functional and kinetic coupling of CK to the sarcoplasmic Ca^{2+}-ATPase ion pump [12, 13] and to the myofibrillar actin-activated myosin ATPase [9, 10, 29, 35, 45, 46]. The improvement by the CK system of the thermodynamic efficiency of ATP hydrolysis is important for some of these pumps, and seems crucial for the sarcoplasmic (SR) Ca^{2+}-ATPase [12, 13]. This SR pump operates with $a\text{-}\Delta G_{transport}$ of approximately 51 kJ/mol (see [44]). The free energy of ATP hydrolysis ($-\Delta G_{ATPhydrolysis}$) at physiological concentrations of ATP (5–8 mM), ADP (10–50 μM) and P_i (5–10 mM) in resting muscle may be estimated to be approximately 55 kJ/mol. This is only slightly larger than $-\Delta G_{transport}$, implying that the SR Ca^{2+} pump operates close to thermodynamic equilibrium [15, 43, 44] and depends very much on a high local ATP/ADP ratio for efficient sequestration of Ca^{2+} into the SR lumen.

Third, an important and seldom recognized consequence of the operation of the CK reaction is the net release of inorganic phosphate (P_i) from PCr. For example, during the first phases of muscle work, P_i increases proportionally to the amount of PCr hydrolysed, while [ATP] and [ADP] levels remain stable [47]. P_i exerts a regulatory effect on glycogenolysis and glycolysis, since it stimulates phosphorylase [48] and phosphofructokinase [49] and additionally relieves the inhibition of hexokinase by glucose-6-phosphate [50]. Thus, in muscle, the availability of P_i may become rate-limiting for glycogenolysis in the absence of PCr hydrolysis, a fact supported by ^{31}P-NMR work (see [51]). A functional coupling of CK with glycolysis, also postulated in our model (Fig. 1) and supported by the colocalization of CK with glycolytic enzymes in muscle [52, 53], could recently be directly demonstrated by ^{31}P-NMR methods in anoxic fish muscle [54].

Fourth, the PCr-circuit also serves as a *spatial energy buffer* or transport system. In this role, PCr is thought to function as an 'energy carrier' connecting sites of energy production, e.g. mitochondrial oxidative phosphorylation, with sites of energy utilization, whereby mitochondrial CK playing an eminent role in this process. This function of CK is supported i) by the specific subcellular compartmentation of the different CK isoenzymes in a variety of tissues, such as muscle, electrocytes, photoreceptor cells, and spermatozoa, the latter two representing highly polar cells (see below and [8]), ii) by evidence indicating subcellular compartmentation of PCr/Cr, ATP/ADP and P_i [29, 55–58], iii) by the localization, structure and functional properties of the Mi-CK octa-

meric molecule which seems very well suited for metabolite channeling [4, 8, 59] and iv) by elegant *in vivo* ^{18}O labelling of the phosphoryl moieties of metabolites in intact diaphragm muscle showing that discrete adenine nucleotide pools exist in these cells and, most importantly, that the rates of appearance of [^{18}O]-PCr are consistent with a CK-catalysed phosphoryl exchange functioning in an obligatory PCr shuttle [58]. Earlier, using a similar isotope technique, the same group provided evidence for compartmentalized *in vivo* catalysis of adenylate kinase activity as well [60]. In connection with the transport function of CK, it is important to note that by speeding up 'communication' between sites of ATP production and ATP consumption, the PCr-circuit model predicts that transitions between different work states are accelerated and smoothened, that oscillations in [ADP] and [ATP] are dampened and that simultaneously the transient times for reaching a new steady-state at a given work load are reduced [4]. This is in line with the fact that in skeletal muscle and cardiomyocytes *in vivo*, the diffusion of adenine nucleotides is severely hindered compared to that of PCr and Cr [29, 61, 62]. Since upon muscle activation, [ATP] remains rather constant and [ADP] rises only little, whereas [PCr] and [Cr] can change drastically [47], the latter compounds are more likely candidates for building up gradients which would speed up trafficking of high-energy phosphates, as a PCr/Cr-shuttle system, from sites of ATP generation to sites of ATP utilization. While the transport function of this PCr-shuttle or PCr-circuit seems to be supported by ample evidence mostly from *in vitro* studies with muscle [6, 8, 18, 34, 36, 63, 64], it turned out very difficult to prove and verify this transport function of CK *in vivo*. Conflicting results have been produced by different research teams (see [35, 36, 42, 65–68]). Whereas in the glucose perfused heart, the flux through the CK reaction, as measured by ^{31}P-NMR, correlates well with the cardiac muscle performance [65, 66], no correlation, or even a negative one, has been reported for skeletal muscle [67]. By contrast, however, a clear correlation of CK-catalysed flux with brain activity has recently been demonstrated on *in vivo* brain, using NMR magnetization transfer [68] (see below). Curiously, *in vivo* ^{31}P-NMR measurements showed that MM-CK deficient transgenic mice [42] or mice expressing less than 30% of normal MM-CK (Dr. B. Wieringa, personal communication), each of which expresses normal levels of Mi-CK, still utilize PCr for muscle contraction. However, inversion transfer measurements revealed no measurable flux through the CK forward reaction [42], indicating that a significant fraction of CK-reaction flux is inaccessible to NMR, or NMR-'invisible'. Interestingly, as soon as the MM-CK levels reached 30–40% or more, suddenly a normal 100% CK-flux was registered as in control animals, indicating that it is mainly the unbound excess of cytosolic CK which is responsible for the measured *in vivo* CK-reaction flux (see [36], Dr. B. Wieringa, personal communication). In light of this unexpected behaviour of the CK-system in NMR terms, the interpretation of earlier CK-flux measurements by ^{31}P-NMR, especially with skeletal muscle, will have to be revisited [36]. At this level of non-invasive *in vivo* measurement, the PCr-shuttle issue is certainly not yet resolved and some surprises may be ahead [36].

In recent years, CK of non-muscle cells, such as spermatozoa, electrocytes, photoreceptor and brain cells, which, like muscle cells, are also characterized by intermittently high and fluctuating energy requirements, has attracted considerable interest. Therefore, the following chapters are meant to give an update on the localization and function of various CK isoenzymes in a variety of non-muscle tissues.

Localization and function of CK isoenzymes in spermatozoa

Vertebrate sperm

Spermatozoa are highly specialized, very polar cells with a DNA-containing head, which may be round or elongated, depending on the species, and which additionally harbours the acrosomal vesicle at its very tip. Adjoining the head is a short midpiece containing mitochondria followed by a very long (50–150 µm) flagellar tail containing the axoneme, basically consisting of microtubules and dynein. The dynein motor protein uses ATP as the direct energy source for the movement of microtubules relative to each other. The flagellar wave thus generated ultimately leads to sperm movement. As shown for muscle [61], it is likely that the delivery of high energy phosphates, such as the rather bulky and negatively charged ATP molecule, from the mitochondria to the distal axoneme, may also be severely diffusion-limited in sperm [69–71]. This limitation may be overcome by the relatively large amounts of CK and total creatine (Cr) present in spermatozoa [70, 71]. In rooster and human sperm, two different types of CK isoenzymes, brain-type B-CK and mitochondrial-type Mi-CK, have been identified by native isoenzyme electrophoresis and

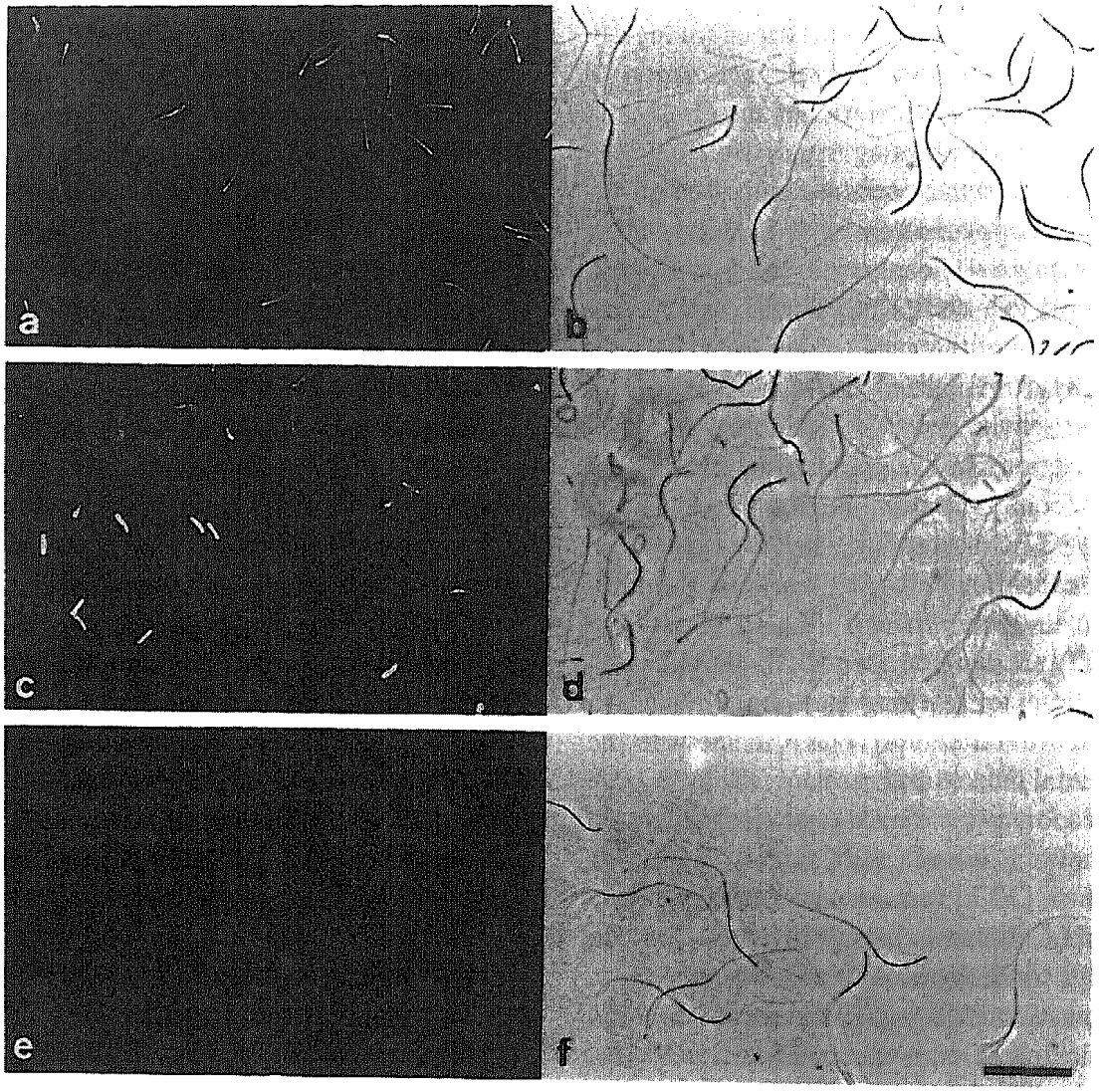

Fig. 2. Compartmentalized localization of creatine kinase isoenzymes in spermatozoa. Indirect immunofluorescence of rooster spermatozoa, stained with specific antibodies against chicken brain-type cytosolic B-CK (a, b); against chicken mitochondrial Mi-CK (c, d); or with preimmune control antibody (e, f), all followed by FITC-conjugated second antibody (Ref. [70]), P. Kaldis *et al.* in preparation). Note the specific staining by anti-B-CK antibodies of the entire sperm tail, but neither of the midpiece nor the sperm head, which in roosters are both rather elongated. The staining intensity with anti-B-CK is often tapering off somewhat towards the distal end of the sperm tail (a). Note also the specific 'box-like' staining by the anti-Mi-CK antibody of the mid-piece where the sperm mitochondria are located (c) and the negative background obtained with pre-immune antibody (e); b, d, and f are the phase contrast images corresponding to the fluorescence pictures a, c and e; bar = 20 µm.

immunoblotting [70]. In addition, by indirect immunofluorescence, B-CK was found along the entire length of rooster sperm tails (Fig. 2a, b), whereas Mi-CK was localized specifically to the rectangular midpiece, just behind the sperm head [70] (Kaldis *et al.* in preparation) (Fig. 2c, d), which, in rooster spermatozoa, is rather elongated (Fig. 2). Thus, an isoenzyme-specific subcellular segregation of CK isoenzymes is seen in these spermatozoa [70, 71]. Most importantly, rooster sperm motility is inhibited by CK blockers [70], indicating a dependence of sperm motility on the CK/PCr-system.

Surprisingly, in a study assessing the fertilizing potential of sperm from infertile male patients, an inverse correlation between sperm CK activity and sperm concentration in the specimens of normospermic and oligospermic infertile men was found [72]. Upon closer examination of sperm morphology, this puzzling fact could be explained by a higher retention of cytoplasm in spermatozoa of infertile men [72]. During normal sperm maturation, excess cytoplasm is lost to the Sertoli cells as residual bodies. Thus, the often observed higher CK content per sperm cell in infertile men can be attributed to the presence of immature or abnormal spermatozoa with irregular head size and retention of cytoplasmic CK due to incomplete sperm development [73]. While the presence in human sperm of brain-type B-CK is not disputed, the question about the identity of the second CK species is not fully settled. According to our study, the second CK species in human sperm is mitochondrial Mi-CK [70] but, according to Dr. Huszar's group [74], it was

suggested to be the cytosolic muscle-type M-isoform of CK. Unexpectedly, the mitochondrial CK isoenzyme present in rooster sperm, which is a non-muscle cell, is sarcomeric muscle-type Mi_b-CK (P. Kaldis *et al.* in preparation). It will be intriguing to see whether, similar to lactate dehydrogenase (for refs see [70]), a possible additional, sperm-specific CK isoform [75] can be identified in human sperm in the future. Independently of the exact nature of this latter CK isoenzyme in human sperm, a low relative ratio of this 'Mi-CK' isoform to B-CK seems to be a good diagnostic sign for a low fertilizing potential of men as shown in a blinded study involving 84 infertile couples [74], indicating that CK and especially 'mitochondrial' CK is a key player also for human sperm function.

Sperm activation and energy metabolism are more complex in vertebrate spermatozoa than in sea urchin sperm [69, 71]. While sea urchin spermatozoa thrive exclusively on mitochondrial respiration by fatty acid oxidation [69, 71], vertebrate spermatozoa are motile also under low-oxygen conditions, deriving the chemical energy additionally from glycolytic pathways [76]. Furthermore, unlike sea urchin sperm, which are released into the sea water as 'dry' sperm, vertebrate spermatozoa are supplemented with relatively large volumes of seminal fluid provided by the seminal vesicles and the accessory prostate gland. The supply of substrates by seminal fluid which contains large amounts of fructose etc. [77] may be relevant for vertebrate sperm motility, since glucose, fructose and mannose were shown to support vertebrate sperm motility under either aerobic or anaerobic conditions [76]. Interestingly, seminal vesicle epithelium cells contain large amounts of PCr and Cr which are released into the seminal vesicle fluid [78]. The accumulation of these compounds in the above cells as well as in the seminal vesicle fluid is regulated by testosterone [79]. Thus, seminal vesicle epithelial cells are the first example of cells that 'secrete' PCr and Cr. An indication that external supply of high energy phosphates to vertebrate sperm via seminal vesicle fluid, representing 50–70% of the seminal plasma, may be important for human sperm motility is corroborated by the fact that upon addition of PCr to human ejaculates or isolated sperms, sperm motility as well as sperm velocity were both enhanced *in vitro* [80]. Blocking rooster sperm CK activity with 10–20 µM 2,4-dinitro-fluorobenzene (DNFB) leads to significant loss of sperm motility [70]. In addition, interference with the delivery of mitochondrial ATP, by blocking the ATP/ADP translocator with 50 µM carboxyatractyloside and at the same time inhibiting glycolysis with 20 mM deoxyglucose, leads to a complete loss of rooster sperm motility. Subsequent addition of 10 mM PCr plus 1 mM ADP, in the presence of 0.5 mM diadenosine-pentaphosphate to block adenylate kinase, led to a significant recovery of *in vitro* sperm motility [70], again indicating that PCr and endogenous CK can support flagellar wave movement.

It is still unclear, however, whether *in vivo*, external PCr in the seminal fluid can be taken up directly by intact sperms and thus be utilized as an immediate energy source, or whether it is metabolized by B-CK, which is also present both in seminal vesicle and prostate fluid [81]. In this latter case, PCr would mediate its positive effects on sperm motility [80] by an indirect mechanism. Based on these results, the controlled interference with the CK system in spermatozoa, either by supplementing CK substrates or by specifically inhibiting CK activity, may turn out to be a valuable tool for the treatment of certain types of male infertility or for male contraception, respectively, in the future. Although statistically significant differences in CK activity, which is exclusively brain-type B-CK, were found in seminal plasma of men with normal spermiograms of oligoasthenozooic sperm, the individual values show an overlap which is too wide for routine diagnostic purposes [82].

Sea urchin sperm

The most convincing evidence for a crucial role of the CK/PCr-system in sperm motility comes from studies with sea urchin sperm. These sperm can be obtained in large quantities in homogeneous form and are reversibly activatable by simply changing the ionic and pH conditions of the sea water [69]. *In vivo*, sea urchin sperms are spawned directly into sea water where they are activated within seconds by sodium influx via a Na^+/H^+-exchanger present in the sperm plasma membrane, leading to an increase in intracellular pH and finally of $[Ca^{2+}]$ [69]. Two different CK isoenzymes have also been observed and isolated from sea urchin spermatozoa [83], a very large unusual tail-CK with a M_r of 145'000 and a multimeric mitochondrial isoform with an apparent subunit M_r of 47'000, which turned out to be an octameric Mi-CK, similar to that of vertebrates ([4], Wyss *et al.*, unpublished). Genetic analysis revealed that the gene for the tail-CK, named according to its location in the sperm tail, contains three contiguous but non-identical CK segments, most likely arisen by two gene duplication events, joined by non-CK-like connectors and

flanked by unique N- and C-termini [84] (see also [85]). The unique tail-CK is a lipophilic protein capable of interacting with sperm membrane preparations [86]. The anchoring of tail-CK to membranes and to phospholipid liposomes *in vitro* [86] is facilitated by myristoylation of the protein at the very N-terminus containing a corresponding consensus sequence [87]. In addition, some evidence suggests that tail-CK may also interact with microtubules of the axoneme [88]. Thus, the isoenzyme-specific segregation of octameric Mi-CK ([4], Wyss *et al.* unpublished) to the sea urchin head and of tail-CK to the cytosol, the plasma membrane and the axoneme, seems to represent a prerequisite for a PCr-shuttle operating also in spermatozoa.

As a matter of fact, evidence obtained by ^{31}P-NMR shows that the large pool of PCr in sperm is depleted or resynthesized, depending on whether sperm motility is induced or inhibited, respectively, by simply altering intracellular pH [89]. This indicates that CK and PCr are indeed directly involved in sperm motility. In a series of elegant experiments with live sea urchin sperm, a direct dependence of sperm motility on an intact CK system could be demonstrated [71]. By specifically blocking CK activity, but not mitochondrial respiration, with low concentrations of 2,4-dinitro-fluoro-benzene (DNFB), the tight coupling of energy utilization and energy production observed in sea urchin sperm is interrupted. Inhibition of CK activity in sperm impedes the transport of high-energy phosphates from the mitochondria to the axoneme and affects the pattern of sperm motility in the manner predicted if energy transport from the sperm head to the tail were diffusionally limited [69]. For example, by computer-assisted analysis of stroboscopic photomicrographs of DNFB-treated and control sea urchin sperm, it was shown that progressive inhibition of CK in these live sperms results in a progressive flagellar wave attenuation, whereby the distal part of the flagellum with the largest distance from the sperm head is affected first and most [90]. In addition to the attenuation of sliding velocity between flagellar microtubules in the distal region of the sperm flagellum, CK-inhibited spermatozoa generate a flagellum bending pattern with shorter wavelengths [90]. These specific alterations of sperm motility, seen after inhibition of CK activity in live sperm, and their reversal upon subsequent addition of ATP following demembranation of the same sperm preparation, provide very strong support for the CK-mediated PCr-shuttle in high energy phosphate transport in spermatozoa, with PCr and Cr being the metabolites channeled along the sperm tail [71]. In our opinion, the whole-cell sperm model is the most convincing 'living proof', so far, for the spatial buffering or energy transport function of the CK/PCr-system proposed in the PCr-circuit model [8, 36, 69–71, 74].

Localization and function of CK in electrocytes of the electric organ of electric fish

Electric fish contain a large bi-lobed electric organ, originally derived from myogenic cells, which is composed of large disc-shaped electrocytes stacked on top of each other to form the many columns of the electric organ. These highly specialized electrocytes, unlike muscle cells, lack a contractile apparatus. Electrocytes are specifically stimulated by the release of acetylcholine from presynaptic nerve terminals of nerves originating in the electric cortex of the fish's midbrain. A large number of nicotinic acetylcholine receptors are present at the ventral, postsynaptic electrocyte membrane. Upon ligand-gated opening of the receptors, sodium ions enter the cells through the receptors, which function as ion channels. The combination of a large number of electrocytes, each approximately 100 µm in height, into many columns enables electric fish to produce a current of sufficient strength to stun or kill their prey or to defend themselves against aggressors. After electric discharge, the intracellular sodium ions are extruded from the electrocytes by the Na^+/K^+-ATPase located within the non-innervated, dorsal membrane face, which is highly invaginated by tubular infoldings, called canniculi. By this way, the surface area of the dorsal membrane system harboring the energy-requiring Na^+/K^+ ion pump is greatly increased (see [91]).

Electric organs of a variety of different electric fish contain high concentrations of CK [92, 93]. The major CK isoform in electric organ is identical to the cytosolic CK found in the skeletal muscle of the same fish, as was confirmed by protein sequencing and cDNA cloning [94–97]. The post-synaptic membrane of *Torpedo* electrocytes is specifically associated with a CK [98] showing a subunit M_r of 43'000, which was also named the 'acetylcholine receptor-rich membrane-associated peripheral υ_2-protein' before it was identified as genuine CK [93, 98, 99]. Based on immunological cross-reactivity with heterologous anti-chicken B-CK antibodies, but not with anti-M-CK antibodies, this protein was first postulated to be brain-type B-CK [99, 100], but was sub-

sequently shown to be genuine M-CK [97, 101]. This discrepancy can now easily be explained, since direct sequence comparison of *Torpedo* electric organ CK with the more than 20 other CK sequences known so far shows sequence motifs in the electric organ CK that are related both to M- and B-type CK's [85]. The above controversy distracted the scientific community from the facts that i) more than one CK isoenzyme is present in *Torpedo* electric organ [100], with the second isoenzyme probably being MB-CK [92, 97], and ii) that CK in electric organ, besides being a soluble cytoplasmic protein, is not only associated as peripheral υ_2-protein with the innervated, postsynaptic acetylcholine receptor-rich membrane [93, 98, 99] but, as shown by immuno-electron microscopy, is also bound to the dorsal non-innervated cannicular plasma membrane face [98] where the ouabain-sensitive Na^+/K^+-ATPase is situated [91]. The colocalization of CK at the dorsal membrane with the Na^+/K^+-pump suggests a role for CK in the regeneration of ATP, via PCr, to fuel the Na^+/K^+-ATPase during recharging of discharged electrocytes ([98], see below). This role serves to keep the local ATP/ADP ratios high in the vicinity of the ion pump, thus increasing the thermodynamic efficiency of ATP hydrolysis and of ion pumping [8, 15, 36, 43, 44]. As in muscle, an additional function of the CK system in electrocytes may be to buffer the intracellular pH, that is, while regenerating the ATP utilized for ion pumping, the CK reaction reutilizes the protons generated by the ATPase reaction [8].

The major, almost exclusive energy utilizing process in electrocytes in the Na^+/K^+-ATPase, which is fully operative after an electric discharge of the electric organ [91]. The aerobic and anaerobic metabolic synthetic pathways in this organ are rather slow [91]. Upon discharge of the electric organ, the high levels of PCr fall rapidly [102], due to the almost exclusive utilization by CK of PCr for the fueling of the fully activated Na^+/K^+-ATPase [91]. This is corroborated by experiments showing that ouabain, a blocker of this ion pump, prevents PCr depletion in discharged electric organ [103]. Most importantly, an intimate functional coupling of CK with the Na^+/K^+-ATPase has been directly demonstrated by *in vivo* saturation transfer ^{31}P-NMR measurements in the resting and stimulated electric organ, showing a highly increased flux through the CK reaction after discharge of the electric organ [103]. Thus in electrocytes, the high energy demands after electric discharge are met at the expense of PCr, and the recovery of the membrane potential during recharging is closely related to the restoration of intracellular PCr levels [102, 103].

In addition, an association between cytosolic CK and synaptic vesicles has been demonstrated in electrocytes of *Torpedo* [98], and the release of acetylcholine from *Torpedo* synaptosomes was shown to be severely affected by inhibition of CK with DNFB [104]. Since mitochondrial content of electrocytes is rather low, and so far, no Mi-CK has been detected in this tissue, a contribution of mitochondrial ATP and/or mitochondrially-derived PCr to the Na^+/K^+-ATPase seems unlikely. However, due to the fact that in electrocytes, the ATP synthetic pathways are slow, but after a discharge, large amounts of energy are needed for recharging, electric organ represents an instructive example for an almost exclusive temporal energy buffer function of the CK/PCr-system, whereas sea urchin sperm represent a similarly instructive example for the spatial buffering or energy transport function postulated in the PCr-circuit model [8, 36].

CK isoenzymes in photoreceptor cells of the retina and in the lens of the eye

Compartmentalized localization of CK isoenzymes in retina

Vertebrate photoreceptor cells of the retina, which are specialized neurons consisting of an outer segment, connected by a thin stalk to the inner segment, the nucleus and synaptic terminations, show a highly polar organization analogous to spermatozoa. The major energy requiring reactions of phototransduction (e.g. the regeneration of cGMP from ATP and GTP, hydrolysed as a consequence of photic stimulation [105]) take place in the outer segments of photoreceptor cells. The distance from the outer segment to the inner segment, where oxidative phosphorylation takes place, can range between 20–50 μm, depending on the species. The highly clustered mitochondria of photoreceptor cells are confined to the ellipsoid portion of the photoreceptor cells' inner segment [38, 106]. Since oxidative phosphorylation is crucial for photoreceptor cell function [107] the question again arises of how high-energy phosphates are transported from the inner segment through the narrow space of the connecting cilium into the outer segment, which is occupied by stacks of photo-sensitive membranes and thus imposes severe diffusion limitations even on small compounds, such as cyclic-GMP [108].

Two isoforms of CK, brain-type (B-CK) and mitochondrial CK (Mi-CK) were found in chicken and bo-

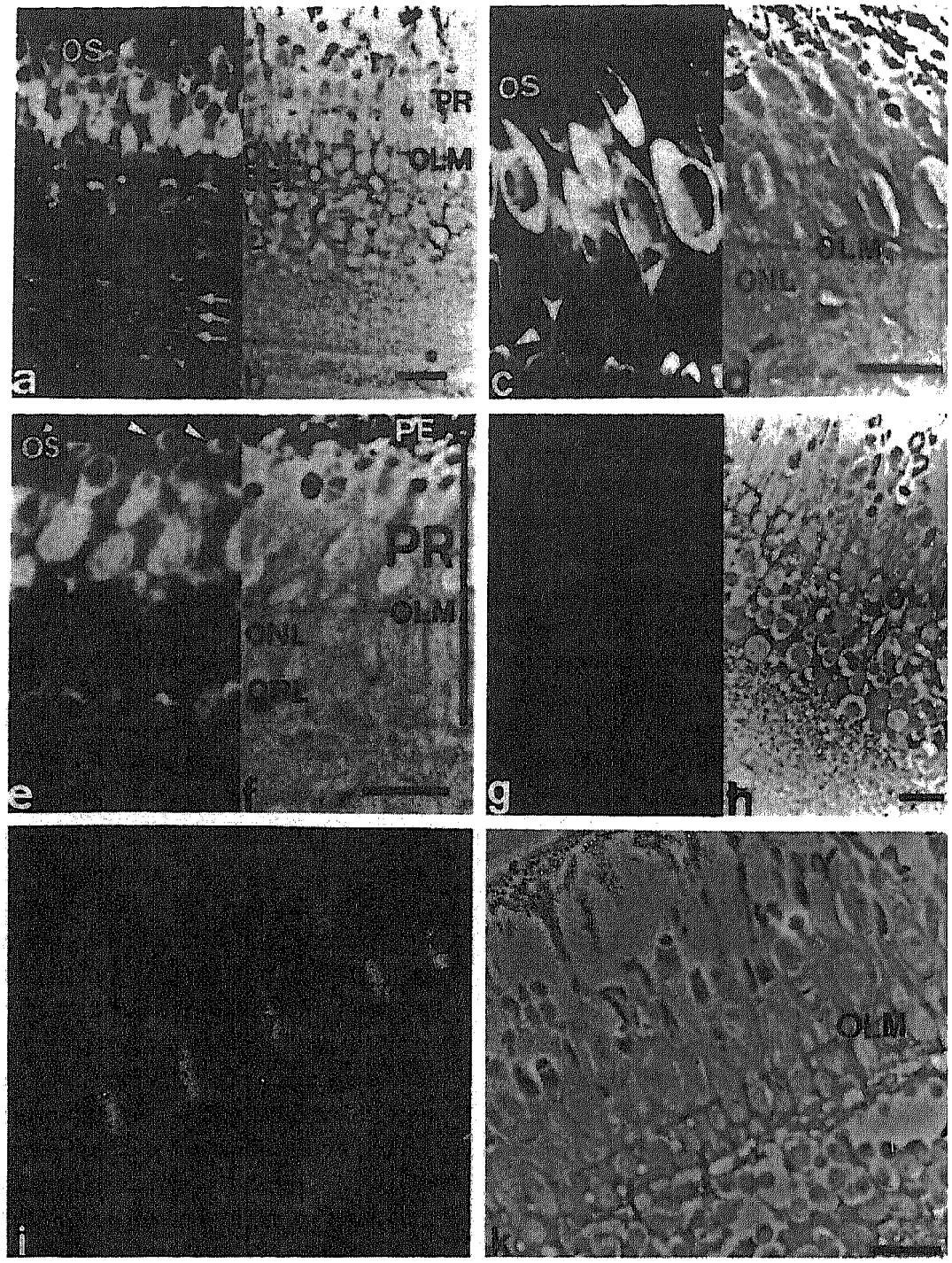

Fig. 3. Immunofluorescence localization of CK isoenzymes in chicken retina: compartmentation of cytosolic and mitochondrial CK in photoreceptor cells. Indirect immunofluorescence of thin cryosections of a retina from adult chicken prepared by the gelatine support technique (for methods see Refs. [109, 110]) stained with specific antibodies against chicken brain-type cytosolic B-CK (a–h) and against mitochondrial Mi-CK (i, k). Fluorescence images (a, c, e, g, i) are displayed with their corresponding phase contrast pictures (b, d, f, h, k). Overview of a chicken retina at low magnification showing anti-B-CK staining of the different retinal layers (a, b). Note that relative to the other cell layers of the retina, the photoreceptor cell layer (PR) is stained most intensely by anti-B-CK antibody, with some stratified staining of the inner plexiform layer (triple arrows in a) and a rather strong staining also of the synaptic region with horizontal cells (arrowhead in c). Somewhat higher magnification depicts strong staining with anti-B-CK antibody of the inner and outer segments of photoreceptor cells (c, d), especially of the myoid portion above the outer limiting membrane (OLM), whereas slightly oblique sections through the photoreceptor cell layer also reveal 'ring-like' immuno-staining of outer segments (OS) (see arrowheads in e), just below the pigmented epithelium (PE). By contrast, mitochondrial Mi-CK localization is entirely restricted to the ellipsoid portion of the inner segment of the photoreceptor cells, where mitochondria are clustered in large numbers (Refs. [38, 107, 110]). Thus on the subcellular level, cytosolic B-CK, present in the inner and outer segment (Ref. [38]), and mitochondrial Mi-CK, present in the inner segment only, are spatially segregated in an isoenzyme-specific manner, thus forming the physical basis for a PCr-circuit in photoreceptor cells (Ref. [38]). PR, photoreceptor cell layer; OS, outer segment, OLM, outer limiting membrane; PE, pigmented epithelium; ONL, outer nuclear layer; OPL, outer plexiform layer; bar = 25 μm.

vine retina [38, 109]. Both of these CK isozymes are expressed at high levels but are distributed differentially in photoreceptor cell inner segments. Mi-CK is restricted to the mitochondria-rich ellipsoid portion, while B-CK is localized both to the ellipsoid and myoid portion (Fig. 3) of chicken inner segments [109].

Although some data from immunofluorescence localization studies [109, 110] (see also Fig. 3), as well as biochemical and physiological measurements [111, 112] suggested that CK is present also in outer segments, unambiguous evidence for the presence of B-CK in photoreceptor cell outer segments, as well as a quantitative analysis thereof, has only been provided recently [38]. The presence of B-CK isoenzyme in rod outer segments (ROS) of chicken retina was initially indicated by the results of immunofluorescence labelling of thin frozen sections of chicken retina (Fig. 3). However, this could only really be corroborated by immuno-gold labelling of bovine retina and has additionally been confirmed by immunoblotting and immunolabeling of isolated bovine ROS, as well as by biochemical characterization of isolated ROS [38]. Thus, while Mi-CK is restricted to the ellipsoid portion of the inner segment, B-CK is present in the inner as well as the outer segment. Therefore, in photoreceptor cells as in spermatozoa, the two CK isoenzymes are in part spatially segregated. The content of creatine kinase in isolated ROS was quantified by measuring creatine kinase activity after membrane disruption with detergent [38]. The ATP regeneration potential provided by the creatine kinase in isolated, washed bovine ROS was 1.2 ± 0.4 IU $\cdot$ mg^{-1} rhodopsin. This value was calculated to be at least an order of magnitude larger than that necessary to replenish the energy required for cGMP resynthesis in ROS, and high enough to regenerate the entire ATP pool of ROS within the time span of a photic cycle (see [38]). Since Mi-CK expression and accumulation in the chicken retina coincide with the functional maturation of the photoreceptors around the time of hatching, this enzyme may represent a good marker for terminal differentiation of these cells [110]. B-CK, on the other hand, is present from early stages of retina development and seems to be relevant for the energetics of retinal cell proliferation, migration and differentiation. The simultaneous expression of both B- and Mi-CK around the time of hatching indicates a coordinated function of the two CK isoforms as constituents of a PCr-circuit in the energetics of vision, which, in autophagous birds like the chicken, has to be operating right after hatching [110].

In photoreceptor cells, which depend on mitochondrial oxidative phosphorylation, ATP produced in inner segment mitochondria may be transphosphorylated by Mi-CK to provide PCr for those fractions of cytosolic CK which are associated specifically with sites of energy consumption, e.g. the Na^+/K^+-ATPase in the plasma membrane of the inner segment or various ATP-requiring processes in the outer segment, where this bound CK regenerates the ATP and maintains high local ATP/ADP ratios. Outer segment CK, which in part may also be associated with the plasma membrane, would play an important role in phototransduction by providing energy for the visual cycle, maintaining high local ATP/ADP ratios and consuming protons produced by ATPases located in the outer segment and thus preventing an acidification of the outer segment compartment (for details see [38]).

The remarkable ATP-regeneration potential provided by B-CK present in ROS indicates that CK-dependent ATP generation may play a major role in many aspects of ATP function in ROS. For example, ATP is not only utilized as an energy source to regenerate cGMP, which is hydrolysed during photic stimulation, but also seems to be a regulatory factor influencing cGMP levels by rapidly quenching light-induced phosphodiesterase activity [113]. This dual role of ATP, as a direct energy source and as a regulatory molecule, has been confirmed by independent groups [114, 115].

Thus, due to its ability to regulate ATP levels in photoreceptor cells, CK in ROS may be involved directly in ATP-mediated regulatory control of phototransduction. Such multiple potential roles of CK in phototransduction, a process that is tightly regulated at many levels, implies that B-CK activity, its intracellular distribution, or even both properties are also likely to be subject to control. A possible regulatory mechanism affecting either of the above properties may be protein phosphorylation. B-CK of rat [116], mouse [117] and chicken [118] are indeed phosphoproteins. The heterogeneity of bovine ROS B-CK observed on 2D gels [38] was similar to that reported for B-CK from other species, suggesting that some of the B-CK in bovine ROS is also phosphorylated. Phosphorylation was shown to alter the kinetic properties of B-CK [117, 118]. This post-translational modification might also regulate the distribution of B-CK between membranes and cytosol, as has been demonstrated for other proteins [119, 120].

CK in the cuboidal epithelium of the eye's lens

Interestingly enough, neonatal rat and human lens were reported to express only a cathodic variant of CK, while near the time of sexual maturation, a dramatic increase in the expression of B-CK and to some extent also of M-CK was noted [121]. It is possible that in lens, as in other tissues such as brain, uterus, placenta, amnion, decidua and mammary gland, B-CK expression is also stimulated by hormones (see [122]). This is supported by the fact that differentiation of lens epithelial cells has been demonstrated in response to vitreous liquid and serum, which both contain hormones [123]. B-CK was localized to the cuboidal epithelial cells of the adult rat lens [121]. These cuboidal epithelial cells, which cover the anterior surface of the lens, have important ion-transport transport functions. Therefore, it is possible that B-CK localized in these cells is functionally associated with areas of high transport ATPase activity.

Localization and functions of CK isoenzymes in brain

CK isoenzymes in brain

Although the relative distribution of CK in different areas or cell types of the brain has been investigated in numerous studies, there is still no consistent and complete overview of the localization of CK isoenzymes in the brain. Regional variations in CK activity with comparably high levels in the cerebellum were reported in studies using native isoenzyme electrophoresis [124] or enzymatic CK activity measurements of either tissue extracts [125] or cultured brain cells [126]. In particular, the molecular layer of the cerebellar cortex contains high levels of CK activity [125, 127], consistent with the recent ^{31}P-NMR findings which indicate that grey matter shows a higher flux through the CK reaction and higher PCr concentrations as compared to white matter [128]. In contrast, high levels of either CK activity or corresponding mRNA were shown in cultured oligodendrocytes [126, 129], typical glial cells of the white matter. B-CK, the major 'cytosolic' CK isoenzyme present in brain [1], has been characterized extensively [118, 130–133]. For chicken B-CK, considerable heterogeneity was found; there are two major B-type subunits and additional subspecies arising from alternative ribosomal initiation [134] and post-translational modifications [118, 132, 135]. Already in the sixties, it was reported that CK activity is associated with brain mitochondria [18, 136]. This enzyme activity was characterized as a genuine brain mitochondrial CK (Mi-CK) [137–139] and later identified as the so-called 'ubiquitous' Mi_a-CK isoform [2, 3, 139]. Mi_a-CK, which was also characterized extensively [4, 139], is localized preferentially as octamers in contact sites of brain mitochondria [20, 31]. In several early studies, the presence of muscle-type M-CK in brain was also postulated (for review see [133, 140]). M-CK has indeed been demonstrated recently in postmortem human brain extracts by biochemical isolation and protein sequencing [141] and in chicken cerebellum by immunoprecipitation, immunoblotting and immunofluorescence analysis [133, 140].

Having a carefully characterized set of highly specific antibodies against chicken CK isoenzymes at hand, the cellular distribution and localization of all chicken CK isoenzymes within the chicken cerebellum was investigated [133]. In addition, the localization, accumulation and developmental appearance of CK isoenzymes during maturation of the rat brain was studied, and these data were correlated with *in vivo* ^{31}P-NMR CK reaction flux measurements [140, 166].

Brain-type creatine kinase isoenzyme in Bergmann glial cells of the cerebellum

The localization of CK isoenzymes is most advanced in cerebellum due to the relatively 'simple', stratified structure of this part of the brain composed of well characterized cell types. Anti-B-CK staining was found in all layers of the cerebellar cortex as well as in the deeper nuclei of the cerebellum, indicating that a high proportion of the cerebellar cell types contain B-CK. The labeling was most intense in Bergmann glial cells (BGC) (Fig. 4a, b, small arrow-heads in b point to BGC cell bodies). The processes of these cells, lying in the vicinity of Purkinje neurons (large arrow), span radially through the entire molecular layer and finally form, with their endfeet, the membrana limitans, which is also stained heavily (Fig. 4a, arrowheads). Thus, the morphology of BGCs is perfectly matched by the intense anti-B-CK staining pattern. Besides BGC, some other cell types in the molecular layer, such as basket cells and neurons in the deeper nuclei, contain B-CK (for details see [133, 140]). Additionally, structures in the granular cell layer, likely to be glomeruli [142] and astrocytes contain significant anti-B-CK immunoreactivity (Fig. 4a, b), whereas cerebellar white matter appears to contain rather low levels of B-

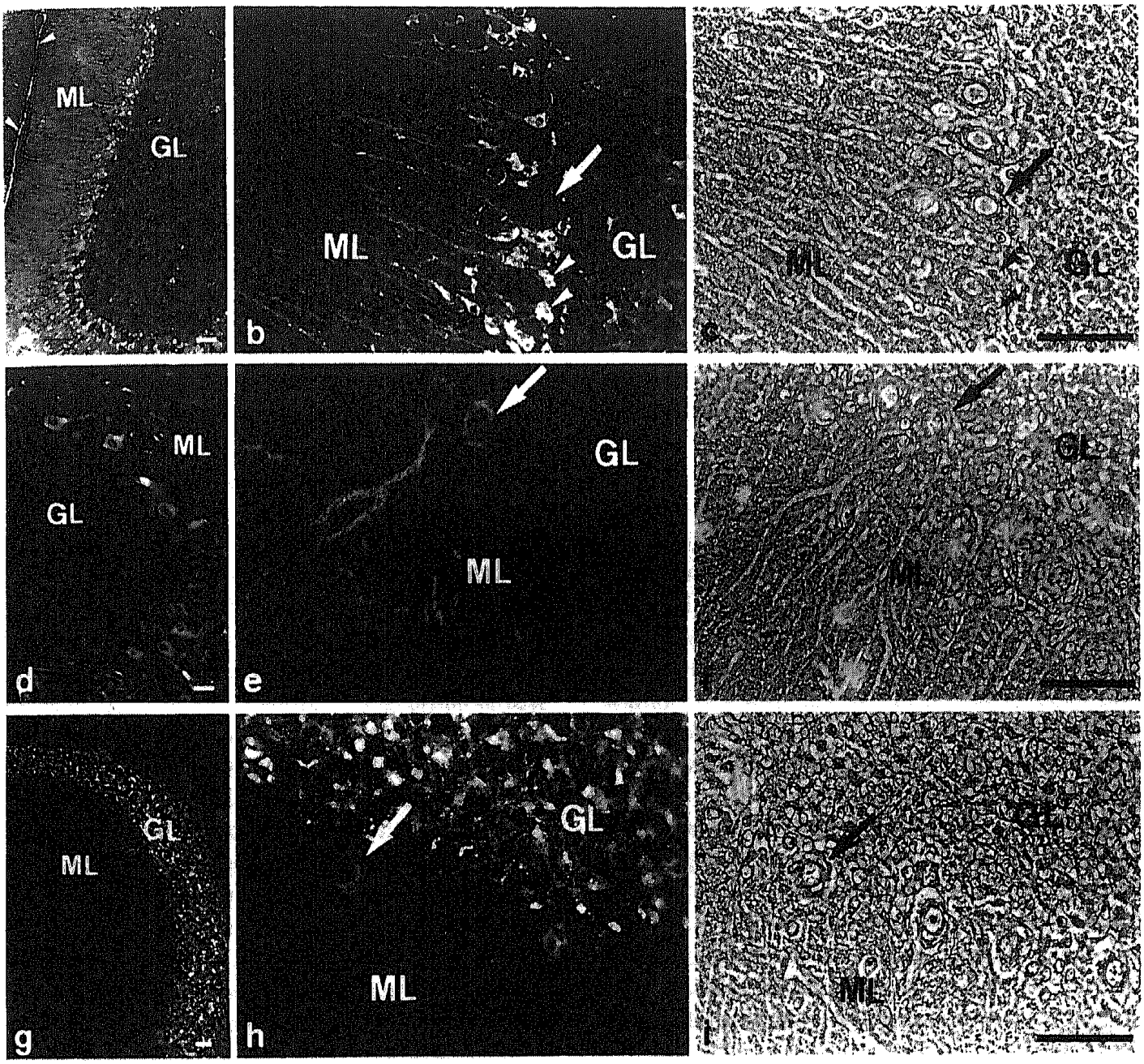

Fig. 4. Localization of brain-type B-CK, muscle-type M-CK and mitochondrial CK in chicken cerebellum. Cerebelli from adult chicken were fixed and embedded in paraffin by standard techniques and labeled by indirect immunofluorescence staining, using specific antibodies against chicken brain-type B-CK, muscle-type M-CK and against ubiquitous mitochondrial Mi_a-CK (for details see [133, 140]). Low magnification immunofluorescence overviews of cerebellar regions (a, d, g), higher magnifications of immunostained pictures (b, e, h), and the corresponding phase contrast pictures in (c, f, i), shown after staining for brain-type B-CK (panels a–c), for muscle type M-CK (panels d–f), and for mitochondrial Mi_a-CK (panels g–i), all followed by rhodamine-conjugated second antibody. Control sections, incubated only with preimmune sera, displayed no significant staining (not shown here, see [133, 140]). Small arrow-heads indicate the cell bodies of Bergmann glial cells (BGCs, in b and e) and the membrana limitans gliae (in a) which are both strongly stained by anti-B-CK antibodies. Large arrows indicate the Purkinje neurons (PNs) which are strongly stained by anti-M-CK antibodies (e and f), weakly stained by anti-Mi_a-CK antibodies (h and i), but remain unstained by anti-B-CK antibodies (b and e). Note that the anti-M-CK staining in the proximal processes of the Purkinje neuron is not uniform, but vesicular (e). This would be consistent with a staining of the endoplasmic reticulum network that is highly enriched in this region of Purkinje neuron. Note also the relatively strong staining by anti-Mi_a-CK of the glomeruli in the granular layer (GL) of the cerebellum. ML and GL refer to molecular and granular cell layers of the cerebellum, respectively; bar = 50 μm. An extensive study concerning the immunohistochemical localization of CK in chicken brain, including more details on the exact localization of the different CK isoenzymes, as well as on the characterization and specificity of the antibodies used was published elsewhere [133].

CK. The latter finding is consistent with previous histochemical and ^{31}P-NMR data [127, 128].

The BGC is a specialyzed type of astroglial cell. It provides the migratory pathway for granule cell migration from the external to the internal granule cell layer during cerebellar development [143, 144]. Another main function of these cells is the proposed ATP-dependent spatial buffering of potassium ions [145, 146], released during the electrical activity of neurons. This function is also reflected by the morphology of BGC, which envelop the synaptic sites of Purkinje neuron dendrites with the exception of the precise sites at which Purkinje spines make contact with parallel or climbing fibers [143]. Since BGC processes directly face the cerebrospinal fuid at the membrana limitans, these cells were suggested to be responsible for releasing the K^+ ions, taken up via ATP-driven Na^+/K^+-ion pumps from the extracellular space around the highly active Purkinje neurons, into the subdural space, which acts as a K^+ sink [145]. It is therefore reasonable to assume that the high B-CK content of BGC (Fig. 4a, b) reflects their high energy demands in relation to spatial K^+ buffering [147]. In this respect, it is interesting to note that Müller cells, representing a functionally and morphologically specialized astrocyte cell type found in the vertebrate retina, were also proposed to be involved in spatial K^+ buffering [145]. Like BGC, the Müller cells also contain significant amounts of B-CK [109]. The presence of B-CK in astrocytes (for details see [126, 133, 140]) may be related to the energy requirements of these cells for metabolic interactions with neurons, e.g. tricarboxylic acid (TCA) cycle metabolite and neurotransmitter trafficking [147].

Muscle-type creatine kinase isoenzymes in Purkinje neurons of the cerebellum

Purkinje neurons play a very important role in brain function. Receiving excitatory input from parallel fibers and climbing fibers, they represent the sole neuronal output structures of the cerebellar cortex. A remarkable feature of Purkinje neurons (PN) is that a single PN makes hundreds of synaptic contacts to a single climbing fiber. Climbing fiber impulses evoke complex Ca^{2+}-spikes and prolongued Ca^{2+}- mediated depolarizations in Purkinje cell dendrites [148] which in turn are thought to play a central role in the mechanism of cerebellar motoric learning [149]. The presence of the 'unusual' muscle-type M-CK (see Fig. 4d,e and [133, 140]) and most likely also of the muscle-type Mi_b-CK (P. Kaldis, personal communication) in Purkinje neurons of the chicken brain may reflect an adaptation of Purkinje neurons to their very special energy requirements. It is known that Purkinje neurons specifically express a whole variety of enzymes involved in Ca^{2+}-homeostasis (for references see [133]). Interestingly, several of these proteins are also muscle-type isoforms, e.g. the skeletal muscle-type ryanodine receptor [150] and calsequestrin, a typical protein of the sarcoplasmic reticulum [151, 152]. In addition, Purkinje neurons contain the highest concentration of sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) found in any non-muscle cell type [153] and also preferentially express a muscle-specific isoform of this enzyme, that is SERCA2b [154]. Recent *in vivo* ^{31}P-NMR saturation transfer experiments, showing that dihydropyridine calcium antagonists reduce the consumption of high-energy phosphates and concomitantly decrease the CK reaction flux in rat brain [155], strongly support the above conclusions that CK is directly or indirectly coupled to energetic processes needed for Ca^{2+} homeostasis or to cellular processes triggered by this second messenger. Thus, the presence of muscle-type M-CK in Purkinje neurons fits well into the general picture that PNs display some 'muscle-like' characteristics and may also reflect the better suitability of M-CK, compared to other cytosolic CK isoenzymes, to associate with certain subcellular structures, e.g. with the endoplasmic reticulum membrane system, in these cells. The vesicular immunofluorescence staining pattern seen in the proximal processes of PNs (Fig. 4e) would be consistent with staining of endoplasmic reticulum vesicles which are prominent in this region of the cell. M-CK was shown in skeletal muscle to be associated with the ATP-dependent Ca^{2+}-pump [12, 13]. Thus, the role M-CK plays in muscle, that is, i) preferentially supplying the Ca^{2+}-pump of the sarcoplasmic reticulum with ATP [12, 13] and ii) keeping local ATP/ADP ratios high in the vicinity of the Ca^{2+}-pump, thereby increasing the thermodynamic efficiency of this ion pump [8, 15, 44], may apply to M-CK bound to the endoplasmic reticulum in Purkinje neurons as well.

Creatine kinase isoenzymes in the glomerular structures of the cerebellum

The granule layer of the cerebellum, especially the glomerular structures, contains high levels of Mi_a-CK as well as B-CK, as judged from the intensities of anti-CK antibody staining (Fig. 4a, b and g, h). These structures,

forming intimate synaptic as well as glial-neuron interactions also called 'neuropils', are known to be rich in mitochondria and to display a very high energy metabolism. Large amounts of energy are needed in these structures for restoration of potassium ion gradients partially broken down during neuronal excitation, as well as for metabolite and neurotransmitter trafficking between glial cells and neurons (for review see [147]). Thus, the localization of both B- and Mi_a-CK isoforms within these structures may be an indication that part of the energy consumed in these giant complexes of mossy fiber, Golgi cell and granule cell synapses (for more details concerning the localization of CK, see [133]), might be provided by a 'PCr-circuit', as it has been proposed for other excitable cells [8, 38, 70, 71].

Creatine kinase in neurons

In brain, B-CK has also been found in association with synaptic vesicles [156], as well as with the plasma membranes [157]. Since a similar association between B-CK and synaptic vesicles as well as the plasma membrane has been demonstrated in electrocytes of *Torpedo* [98], the electrocyte system serves as a good analogy for the function of this portion of B-CK in brain. Convincing data concerning a direct functional coupling of CK with the Na^+/K^+-ATPase have been obtained by *in vivo* ^{31}P-NMR studies on electric fish, which showed that CK and the membrane-bound Na^+/K^+-ATPase are tightly coupled in the resting as well as in the stimulated electric organ [103]. Additionally, CK bound to synaptic vesicles in electrocytes is involved in neurotransmitter release [104]. Thus, the fractions of CK that are bound to synaptic vesicles and to the plasma membrane in neurons may also be involved in neurotransmitter release, as well as in the maintenance of membrane potentials and the restoration of ion gradients before and after electrical discharge, both in conjunction with the Na^+/K^+-ATPase [68, 103]. This is consistent with the fact that high energy turnover and, concomitantly, high CK concentrations have been found in those regions of the brain that are rich in synaptic connections, e.g. in the molecular layer of the cerebellum, in the glomerular structures of the granule layer and also in the hippocampus (Hemmer, unpublished observation). In neurons, the Na^+-extrusion activity facilitated via the neuron-specific Na^+/K^+-ATPase [158] is especially high in the synaptic region (see [159]). This is also true for Ca^{2+}-extrusion activity, mediated either via the plasma membrane Ca^{2+}-pump responsible for net extrusion of calcium out of neurons, or via the Na^+/Ca^{2+}-exchanger. The Na^+/Ca^{2+}-exchange is driven by the Na^+ gradient which in turn is maintained by ATP indirectly through the operation of the Na^+/K^+-ATPase (for review see [160]). The observation that a rise in CK levels, observed in a fraction of brain containing nerve endings and synapses, parallels the neonatal increase in Na^+/K^+ ATPase is also suggestive that higher levels of PCr and CK are characteristic of regions in which energy expenditure for processes such as ion pumping are large [159]. In addition, protein phosphorylation which plays an important role in brain function is also thought to consume a sizable fraction of the total energy available to these cells [159].

Finally, 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 [161, 162]. 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 neurons. In addition, during preparation of neuron-specific enolase, brain CK co-chromatographes with the latter glycolytic enzyme [163], indicating a functional coupling of brain CK with glycolysis as was demonstrated in muscle [8, 52–54].

Interestingly, in differentiating primary cell cultures of neuronal cells, some B-CK was localized to the nuclei [126], whereas in adult chicken and rat brain, after *in situ* immunofluorescence staining with our anti-CK antibodies [133, 140], the nuclei of most neuronal cells, which are, however, fully differentiated, remained unstained.

Postnatal accumulation of mitochondrial CK in rat brain and in vivo *function of CK*

In the altricial neonates (mouse, rat, rabbit, pig and human), marked quantitative and qualitative changes in the physiology of ATP metabolism occur postnatally (see [164]). Similarly, rather dramatic postnatal increases in total CK activity and PCr content were noted. 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 increases 4-fold, as measured by saturation transfer ^{31}P-NMR [165, 166], ii) the brain develops the capacity to increase ATP synthesis by oxidative phosphorylation in response to

sudden changes in energy demand [164] and iii) a population of cerebral brain mitochondria appears with tight contacts between inner and outer membranes [165]. Since Mi-CK has been identified in isolated contact site membrane fractions of brain mitochondria [20, 31] and since octameric Mi-CK was shown to be able to induce contact formation between isolated mitochondrial membranes [25], the appearance of the population of mitochondria described above may be related to the expression and accumulation of Mi-CK in these mitochondria. In rat brain, an increase of Mi-CK activity by 4–6 fold, which is higher than the increase of total CK or B-CK activity over this time period, takes place between days 12–20 of postnatal development, concomitant with a corresponding 4 fold increase in the *in vivo* rate of CK-catalysed reaction flux [166]. These observations, showing that the developmental appearance of Mi-CK parallels the maturational changes in brain energy metabolism, suggest that Mi-CK, and CK in general, are critical in the control of cellular ATP metabolism in the adult brain [166]. The fact that in the developing brain, B-CK expression is acutely stimulated by vitamin D metabolites, but not by estrogen [167, 168] is certainly interesting in the context of brain development.

The interpretation that CK plays a key role in the energetics of the adult brain is supported by very recent *in vivo* ^{31}P-NMR magnetization transfer measurements showing that the pseudo first-order rate constant of the CK reaction (in the direction of ATP synthesis) as well as the CK flux (J_f) correlate with brain activity, which was measured by EEG as well as by the amount of deoxy-glucose phosphate formed in the brain after administration of deoxy-glucose [68]. These data show that *in vivo* the CK/PCr-system serves not merely as a temporal energy buffer [37], but has also a spatial energy buffer or transport function [8] with Mi-CK functioning as a key player in the intricate energy distribution system [4, 8] also in brain [166].

Localization and function of CK in uterus and placenta

Two major CK isoenzymes, B-CK [169] and ubiquitous Mi-CK, have been identified both in uterus and placenta [170]. In addition, a recent analysis of the myometrium of gravid guinea pigs revealed, besides the prevalence of BB-CK and the relatively low concentration of Mi-CK, the presence of MB- and MM-CK in this tissue [171]. During pregnancy, the total CK activity in myometrium increases almost by a factor of three, but the distribution of isoenzymes does not significantly change with gestation, except that the contribution of Mi-CK increases from trace activity in the non-gravid uterus to 5%! in the gravid uterus [171], which is indicative for a role of Mi-CK in the gravid myometrium. In detergent skinned fibres from myometrium, significant amounts of BB-CK, but not MM- or MB-CK, remain specifically bound [171], indicating an isoenzyme-specific compartmentation of CK in myometrium, with a fraction of BB-CK located at the myofilaments. Contraction experiments with chemically skinned fibres showed that in the presence of 10 mM PCr plus 250 μM ADP, in the absence of ATP, non-gravid and gravid uterine fibres are able to support 43% and 65% of maximal tension, respectively, via the endogenous CK system [171].

These results indicate that the presence of B-CK at the myofilaments and of Mi-CK in mitochondria, as well as their elevated specific activity during pregnancy, will lead to better interrelation and coupling between oxidative metabolism and force of contraction during increased demand for parturition [171]. This notion is also supported by the fact that *in vivo*, the uterine PCr levels increase significantly before labour, when large metabolic demands are made on the contracting myometrium. [PCr] remains high during parturition and returns to the much lower prepartum levels within a week after birth [172].

The structural and functional reorganization of the gestating uterus is under hormonal control. As a matter of fact, B-CK is *the* major estrogen-induced protein in uterus [173], and by CAT-assays, a functional estrogen enhancer was demonstrated to be contained within a 2.9 kb fragment of the 5'-upstream flanking region of the B-CK gene [174]. Very recently, it has been reported that the expression of both B-CK and Mi-CK in uterus as well as placenta are in fact hormone-regulated [170] and that both CK isoforms can be induced very rapidly by estrogen *in vivo* and *in vitro* [168, 170, 173, 175]. *In vivo*, B-CK and Mi-CK expression are highly coordinated both with respect to time during pregnancy and after delivery [170]. Interestingly, the hormone-induced increase in specific CK activity in experimentally estrogen-stimulated rat uterus is paralleled by changes in uterine PCr concentration [176]. Most importantly, this increase, partly due to an upregulation of Mi-CK as well, is paralleled by an increase in CK reaction flux from PCr to γ-ATP [177]. Taken together, these results suggest an important role of the PCr/CK system in uterus and pla-

centa for the maintenance and termination of pregnancy [171].

Localization and function of CK in intestinal brush border epithelial cells and in endothelial cells

CK-supported contraction of intestinal brush-borders

In intestinal epithelial cells, B-CK and Mi-CK were also found to be compartmentalized subcellularly. While B-CK is distributed diffusely throughout the cytoplasm of these cells, it is concentrated distinctly in the brush border terminal web region, where the contractile-ring myosin is located. The mitochondrial Mi-CK isoenzyme, on the other hand, is specifically confined to the mitochondria just subjacent to the terminal web region [178].

Glycerol-permeabilized brush borders, with their cytoskeleton intact retain significant amounts of B-CK, which is indicative for a relatively strong subcellular association of a fraction of the enzyme with internal structures of brush borders [178]. Such brush border preparations do contract *in vitro* by virtue of their circumferential ring myosin, and the extent of contraction can be measured by the curvature of the brush borders at the level of the circumferential ring. Maximal contraction can be supported equally well either by an externally added ATP-regeneration system [phosphoenol-pyruvate (PEP)/pyruvate kinase] or by simply adding PCr in the presence of micromolar concentrations of ADP [178]. This indicates that endogenous B-CK is sufficient for delivery of ATP to the contractile-ring myosin and for ATP regeneration. Most importantly, whereas PEP-pyruvate-kinase-supported contraction is efficiently blocked by external addition of an ATP trap (hexokinase and glucose), the PCr-ADP-supported contraction is not at all inhibited [179], thus demonstrating that ATP is preferentially supplied, via PCr, to the circumferential-ring myosin by endogenous CK bound at the terminal web. This preferential supply of ATP imparts to this myosin a selective energetic advantage over other cellular ATPases. Thus, similar to muscle, CK-coupled contractile-ring myosin appears to be one end of an energy circuit that supplies the energy for brush border contraction [179].

CK in epithelial and hair cells of the inner ear

The organ of Corti and the stria of the inner ear are known to contain high concentrations of CK [180], possibly involved in the energetics of ion transport and auditory sensation. By immuno-histochemical analysis, marginal cells of the cochlear stria vascularis, as well as dark cells and transitional cells of the vestibular system, all contain an abundance of CK [181]. These cell types concentrate K^+ in the endolymph of the inner ear against a large gradient and depend, for such ion transport, on a ouabain-sensitive basolateral Na^+/K^+-ATPase. Thus, in analogy to the well-known cases in other cells, it is reasonable to speculate that CK in strial marginal cells and dark cells is also coupled to this ion pump and maintains a thermodynamically favourable ATP/ADP ratio in the vicinity of the pump. High levels of CK have also been demonstrated in the cochlea's inner and outer phalangeal (Deiter's) cells and, although at lower levels, in the sensory hair cells [181]. CK in Deiter's cells may have a function in the re-uptake of K^+-effluxing from the sensory hair cells analogous to the role played by CK in Bergmann glia cells and Müller cells of brain and retina, respectively (see previous chapters).

CK in endothelial cells

Endothelial cells lining the inside of blood vessels, when maintained under normoxic conditions, express various CK isoenzymes (BB-, MM- and Mi-CK) and possess significant stores of PCr [182]. If these cells are exposed for prolonged periods of time to hypoxic conditions, they exhibit a significant reduction in their PCr stores and dramatically upregulate their glucose transport activity [182]. Thus, even though very little is known about the function of CK in these cells, endothelial cells obviously seem to require PCr to meet their metabolic demands, especially under metabolic stress. It will be interesting in the future to localize the CK isoenzymes at the subcellular level in endothelial cells and to establish functional assays to probe for CK function.

CK in kidney and rectal salt gland

Kidney

PCr and CK have received almost no attention in kidney, probably because the levels in whole kidney are ve-

ry low as compared to muscle and brain. For example, CK activity in kidney compared to muscle is lower by at least a factor of 100 [183]. However, by microdissection of different regions of the kidney, it was shown that [PCr] and CK activity vary by as much as 5–10-fold and 100-fold, respectively, depending on the segments along the nephron. The distal convoluted tubules containing highest [PCr] and specific CK activity, with the latter reaching some 5% and 20% of muscle and brain CK activity, respectively [183]. Thus, it emerged that PCr and CK must be confined to a few cell types of the whole kidney. The first results obtained with immuno-peroxidase staining for CK in kidney were in agreement with this notion in that CK was mainly confined to the epithelial cells of the thick ascending limb of the Henle's loop and the collecting tubules [184]. In a subsequent study, using isoenzyme-specific anti-CK peptide antibodies, two CK isoenzymes, brain-type B-CK and ubiquitous Mi-CK were identified and characterized [185]. By immunohistochemical staining, it was demonstrated that both CK isoforms are co-localized on a cellular level in the inner stripe of the outer medulla of rat kidney, mainly confined to the distal tubules of nephrons [185]. Interestingly, this distribution of CK in kidney corresponds in general to the region of greatest ATP utilization, oxygen consumption and sodium transport by the Na^+/K^+-ATPase [183]. Thus, besides providing an energy buffer during periods of low oxygen tension, characteristic of the renal medulla, the cellular co-localization of the two CK isoforms in a region of high energy turnover related to ion transport indicates a more active role for the CK/PCr-system in the distal nephron [185], e.g. it may serve as an energy shuttle system to provide energy for sodium transport and may in addition fulfil other CK functions postulated in the PCr-circuit model [8]. This is supported by the fact that upon hypoxia of the kidney, similar to muscle, electric organ, brain and macrophages (see this article), PCr in the distal nephron is also depleted more rapidly and to a greater extent than ATP [183].

Rectal salt gland

The highly specialized rectal gland for sodium excretion, found in elasmobranchs such as dogfish and sharks, was shown to contain very high levels of PCr, and high expression levels of brain-type B-CK as well as of ubiquitous Mi-CK [186]. This CK isoform composition is the same as in mammalian kidney [186]. As in kidney, these two isoforms are colocalized on a cellular level and are found to be concentrated at the basal region of the tubule cells where the sodium transport ATPase is also located [186]. Thus, the two CK isoforms in these sodium-secreting tubule cells may provide the components of a shuttle system for the regeneration of energy required for sodium transport and may keep the thermodynamic efficiency of this process high. Such a role is corroborated by the fact that stimulation of rectal gland sodium-secretion by cyclic AMP causes a rapid decrease in PCr levels with little or no change in [ATP] [187], suggesting that sodium-excretion in this salt gland is tightly coupled to PCr-hydrolysis in order to maintain high local ATP/ADP ratios in the vicinity of the ion pump.

CK in adipose tissue

Brown and white adipose tissue both contain PCr and CK activity, with the specific activity of CK in brown fat being approximately 50 times higher than in white fat tissue [188]. In brown adipose tissue, which is responsible for heat generation through a process called 'non-shivering thermogenesis', the CK activity is in the same order of magnitude as that found in cardiac or nerve tissue. So far, the CK isoenzymes in this tissue have not been identified, but the function of CK may be directly related to thermogenesis. As a matter of fact, based on *in vivo* Cr-uptake experiments, it was shown that the labelling of the total Cr pool with radioactive Cr proceeded much faster in adipose tissue than in skeletal muscle [188]. It was concluded that fatty acids and free Cr may be synergistic in promoting mitochondrial respiration for thermogenesis [188].

CK in pancreas

The possible importance of CK in endocrine tissues may have been overlooked in the past. The fact that PCr [189] and CK activity [190] were identified in the islets of Langerhans of pancreas, with B-CK as the major CK isoenzyme [190], indicates that PCr and CK may play a role in the secretion of insulin and/or glucagon. In pancreas, the energy required for insulin exocytosis was assumed to be supplied by ATP synthesized in acinar cells. However, ^{31}P-NMR measurements of cerulein-stimulated rat pancreas indicate i) that ATP for insulin exocytosis was derived from phosphorylation of ADP via PCr by pancreatic CK and ii) that large amounts of PCr are synthesized during the first minutes after cerulein-stimula-

tion [191]. During this process, the PCr levels reach a maximum at 10 min, then fall between 10–20 min after stimulation and finally return to control levels [191].

CK in thymus, thyroid and liver

In addition to expressing variable amounts of B-CK, the thymus was shown, quite surprisingly, to contain also muscle-specific M-CK [192], as well as another muscle-specific protein, the 165'000 M_r M-band protein. Nodules, embedded in the thymic reticulum, stain *in situ* positively for both of these proteins. If thymus tissue was dissociated into single cells and cultured at high density, myotubes which were morphologically similar to those from muscle-derived cultures appeared and stained positively for the two muscle proteins mentioned above [192]. Obviously, in the thymus, there are some myoid cells present which are capable of forming myotubes. This explains the occurrence of muscle-specific proteins in this tissue.

Already very early, the presence of CK in thyroid has been reported, and the isoenzymes found in this tissue have been tentatively identified as MM- (MB-) and BB-CK [193]. The invariable presence of significant amounts of CK in the thyroid of several species including man suggests that this enzyme may have a role in thyroid tissue metabolism or hormone biosynthesis, however, no follow-up study on the localization and function of this enzyme in this tissue has been published.

Surprisingly, the presence in human liver of mitochondrial CK, showing electrophoretic properties similar to those of human cardiac Mi-CK, has also been reported, and Mi-CK was actually purified from the mitochondrial fraction of human liver [194]. By contrast, all our efforts by native CK isoenzyme analysis, affinity labelling of CK with radioactive N-bromoacethyl-3,3',5-triiodo-L-thyronine, immunoblotting of extracts from whole liver tissue or from highly enriched mitochondrial fractions from chicken, mouse and rat livers [195], as well as Northern-blot analysis of chicken liver with Mi-CK-specific cDNA probes [M. Stolz, IZB, ETH, personal communication], did not reveal Mi-CK in liver. If anything, only minute amounts of B-CK, which easily could have been derived from blood vessels (smooth muscle, and endothelial cells) or blood cells (macrophages and blood platelets) have occasionally been found in this tissue. This raises the interesting question of whether human liver is an exception with respect to CK content or whether the expression of Mi-CK in human livers, as described by the above authors, may be linked to a specific unrecognized disease affecting the "healthy" patient whose liver was taken post-mortem.

CK in cartilage and bone

Cartilage cells

The presence of PCr in chondrocytes was demonstrated by ^{31}P-NMR measurements of superfused resting zone cartilage from the growth plates of bones from young animals [196]. Extraction and chemical analysis revealed the highest amounts of PCr in the proliferative region of cartilage, but no PCr is present in calcified cartilage [197]. In sharp contrast to developing skeletal muscle, where a transition from BB-CK in embryonic to MM-CK in adult muscle takes place, exclusively MM-CK was found in resting and proliferating cartilage, while MB and BB-CK are clearly the predominant CK's in hypertrophic cartilage [197]. The levels of CK activity are directly related to chondrocyte maturation, with CK activity increasing with the progression of chondrocyte hypertrophy [197]. As in uterus, the expression of B-CK in cartilage cell cultures is regulated by estrogen [198] and also by calciotrophic agents, such as certain vitamin D metabolites [199]. Most importantly, lowering of the energy status of developing cartilage and bone *in vivo*, either by feeding of rats with the creatine analogue 3-guanidino-propionic acid (GPA), or by addition of GPA to cultured chondrocytes *in vitro*, leads to a significant inhibition of normal cartilage development and differentiation, respectively [200].

Bone cells

B-CK activity in bone cells in culture is also stimulated, like in cartilage cells, by some, but not all vitamin D metabolites [201] and, in addition, by parathyroid hormone and prostaglandin E_2 [202]. Most intriguing, however, is the direct and sex-specific stimulation of B-CK expression by sex steroids in rat bone [203]. Whereas in bones of female rats, 17β-estradiol (E_2), but not testosterone, stimulates the appearance of B-CK activity, the inverse situation is observed in bones of male rats, that is, testosterone stimulates B-CK synthesis, while E_2 is ineffective. This indicates that gonadal steroids may contribute to stimulating bone growth and to maintaining a bal-

anced bone-turnover, with CK being directly involved in the energetics of these processes [200].

Very recently Ch'ng and Ibrahim (1994) [225] have shown that when a rat osteoblastic cell line is induced to differentiate with 1,25-dihydroxyvitamin D_3, the expression of B-CK is upregulated by a two-fold increase in the transcription rate and by translational modulation incolving increased B-CK mRNA stability as well as binding of a cytosolic factor to the highly conserved 3′-untranslated region of the B-CK mRNA [225]. The transacting factor shown to prevent completion of translation of the mRNA [226] may prevent diffuse and indiscriminant expression of B-CK in the cell and target the protein-RNA-complex to the appropriate subcellular compartment for local expression of B-CK [225]. Most importantly, inhibition of the up-regulation of B-CK in osteoblasts with anti-sense RNA targeted to the B-CK mRNA results in failure to attain the mature osteoblastic/osteocytic phenotype (Ujihara and Ch'ng, manuscript in preparation).

The importance of CK in eneragy metabolism is generally reflected by the stringent regulation of its expression, both developmentally and spatially. The molecular melchanisms, both at the transcriptional and translational level, of B-CK expression in osteoblasts obviously play significant roles in osteoblast-osteocyte physiology which is characterized by changes in cytoskeleton and increased activity of membrane pumps in differentiated osteoblasts-osteocytes [225].

CK in macrophages blood platelets

CK was first identified in phagocytic white blood cells, that is, in rabbit alveolar macrophages, by DeChatelet *et al.* in 1973 [204]. Later, it was found that mouse macrophages contain B-CK as the major CK isoenzyme, whereas the same *human* cells seem to express B-CK and possibly also Mi-CK [205]. The expression of CK in mouse and human mononuclear phagocytes is a developmentally regulated process occurring during *in vivo* and *in vitro* differentiation of monocytes into macrophages [205]. The latter cells display relatively high specific CK activity and accumulate PCr in 3–5-fold molar excess over ATP [205]. The induction of CK during differentiation of monocytes into macrophages in cell culture occurs independently of the concentration of Cr in the medium. The size of the intracellular PCr pool in macrophages, however, is directly proportional to the [Cr] of the culture medium [206]. Macrophage differentiation is also associated with a marked increase in the Cr transporting capacity [205]. This is evident that macrophages, like muscle and some neurons, do not synthesize their own Cr, but accumulate the compound via a transmembrane Cr-transporter that has recently been cloned from rabbit brain and functionally expressed in COS-7 cells [207]. Most important, however, is the finding that during phagocytosis in both resident and thioglycollate-elicited mouse peritoneal macrophages, ATP levels remain fairly stable, while PCr concentration decreases by 40–50% [208]. These results demonstrate that the ATP consumed by macrophages during phagocytosis is replenished by CK via PCr.

B-CK [209] as well as PCr [210] were identified also in isolated, washed blood platelets which can therefore be a possible source of CK in blood plasma and serum. The fact that aerobically generated ATP preferentially disappears from blood platelets during thrombin-induced aggregation and clotting [211] would indicate that mitochondrial CK, although not identified in these cells so far, may also be contained in blood platelets and that CK may have a function in the above processes.

CK in malignant tumors and cancer cells

As shown above, brain-type B-CK is a major enzyme of cellular energy metabolism in non-muscle cells. In this context, it is interesting that B-CK is overexpressed in a wide range of solid tumors and tumor cell lines. For example, highly elevated levels of this enzyme have been detected in tumor samples obtained from patients with small-cell lung carcinoma, colon and rectal adenocarcinoma, breast and prostate carcinoma, as well as neuroblastoma [212–216]. As a result of its elevated level, B-CK can be used as a diagnostic marker for small-cell lung carcinoma [213].

In many cancer patients with one of the carcinomas mentioned above, B-CK, and in certain cases also Mi-CK, are released into the serum, whereby elevated levels of CK in tumors and/or in the serum [217] are associated with untreated, progressive or metastatic disease. Thus, highly elevated levels of CK in the serum of such patients seem to be an adverse prognostic indicator (for refs. see [4, 218, 219]). Most likely, a rather bulky tumor or an advanced stage of malignancy is required for the continuous release of routinely detectable CK into the serum [215]. The presence of Mi-CK in the serum of cancer patients with a variety of adenocarcinomas [220] and

with certain melanomas [221] was suggested to be a reliable tumor marker, with Mi-CK-positive individuals showing significantly higher mortality rates compared to Mi-CK-negative ones [4, 222].

As mentioned above, B-CK is an estrogen-regulated protein in uterus, placenta and other specialized cells. This holds true also for human breast cancer explants and cultured breast cancer cells that are estrogen-induced. In such tumors and cells, a positive relationship between B-CK and estrogen receptor content has been observed [216]. This indicates that a quantitative evaluation of B-CK activity in human breast cancer may be of potential value for the detection of hormone-responsive tumors, as well as for the strategy of treatment.

It seems that increased amounts of PCr are used by a variety of malignant cells as an energy source. This is corroborated by the fact that malignant cells often contain highly elevated [PCr] and that a broad spectrum of cancer cells derived from different solid tumors is growth-inhibited by the creatine analogue, cyclo-creatine (cCr) [218, 219]. It was shown that in these cells, cCr accumulates as phosphoryl-cCr (PcCr), with thermodynamic and kinetic properties different from those of PCr. In tumor cells that express high levels of CK, the tumor-growth inhibition is seen at extracellular concentrations of cCr ranging between 2–8 mM, which led to an intracellular accumulation of the compound reaching 30–50 mM [219], indicating that the active form may be PcCr. By contrast, tumor cell lines expressing low levels of CK accumulate much less PcCr and, consequently, are growth-inhibited only at 1-fold higher concentrations of this Cr analogue [219]. However, the fact that Cr itself, although only when fed to cancer-bearing rats at higher concentrations than cCr (up to 5% of the total diet), shows a similar inhibitory effect on tumor growth *in vivo* [218], argues against the proposition that cCr inhibits tumor growth by disturbing the energy metabolism. Therefore, the question remains of whether the observed inhibition of tumor growth rates by cCr and especially by Cr is due a direct disturbance of energy metabolism by the respective phosphagens, PcCr and PCr, in tumor suppression, or to effects of the phosphagens on other cellular processes.

Most interesting with respect to a possible CK/cancer connection is the observation that B-CK gene expression is induced by adenovirus type 5 [223]. It was shown that the 5′-upstream promotor of the human B-CK gene has a strong similarity to the adenovirus promotor for the E2E gene, the latter encoding a 72 kDa single-stranded-DNA-binding protein which is crucial for virus replication. The expression of the E2E gene is highly dependent on the adenovirus products of the Ela region coding for two major oncogenic nuclear phosphoproteins (for refs. see [223]). As it turned out, both B-CK activity and B-CK mRNA levels are highly induced by the oncogenic products of the adenovirus Ela gene region [223]. These very exciting results suggest that the control of B-CK expression lies along the path that is closely linked to cell growth control. The induction by an oncogene of a cellular gene for energy metabolism, such as B-CK, may be of significance for the metabolic events that take place after oncogenic activation [223] and thus may turn out to be relevant for the etiology of the transformed phenotype of cancer cells which are known to be able switch from one energy-producing pathway to another. For additional information concerning the identification of CK isoenzymes in normal and diseased non-muscle tissues and cells of man, the reader is referred to references [214, 224].

In conclusion, the 'ubiquitous' presence of B-CK in many non-muscle tissues, with B-CK in most cases being co-expressed with ubiquituous Mi_u-CK, seems to indicate a multitude of functions for the CK system in these tissues and cells. In some of the very specialized tissues or cells, like sperm, electric organ and photoreceptor cells, the identification of the isoenzymes, their subcellular localization, as well as some of the functions of the CK/PCr-system have been elaborated quite extensively. In these well studied tissues and cells, the respective functions of CK are more obvious than in other, less specialized tissues and cells. It will be extremely intriguing to see the phenotypes of transgenic mice with the B-CK and/or Mi_u-CK genes knocked-out, if such null mutants should turn out viable (for the phenotype of M-CK null mutant transgenic mice see [42]). If our hypotheses of the CK/PCr-systems are correct, one would expect to see some severe phenotypes in B-CK knock-out mice. For example, sperm motility (fertility), vision, brain development and brain functions would all be expected to be severely impaired, unless compensatory measures are taken by the affected tissues and cells, as has often be seen in the recent past with gene-targeted knock-out mice. However, for this sort of work, well-trained organ and cell physiologists, being able to test subtle physiological differences in transgenic versus normal mice, will be in great demand again in the near future. Thus, sophisticated molecular biology and organ physiology shall embrace each other more closely than ever before and, in a combined effort, will help to solve some of the puzzles of life.

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