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High content of creatine kinase in chicken retina: Compartmentalized localization of creatine kinase isoenzymes in photoreceptor cells

(retina/creatine phosphate shuttle)

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ABSTRACT **Two isoforms of creatine kinase (CK; ATP:creatine *N*-phosphotransferase, E.C. 2.7.3.2), brain type (BB-CK) and mitochondrial type (MiMi-CK), but not the muscle types (MM- or hybrid MB-CK), were identified by cellulose polyacetate electrophoresis and immunoblots in retina from adult chickens. Indirect immunofluorescence labeling of cryosections of retinas revealed high concentrations of BB-CK in both rod and cone photoreceptor cells. Most of the fluorescence staining with anti-B-CK antibodies was found within the myoid and the ellipsoid portions of inner segments and the peripheral region of the outer segments. Significant staining with anti-B-CK antibodies was also found in horizontal cells and in the optical nerve fibers, with additional stratified staining in the inner plexiform layer. MiMi-CK was solely demonstrated in the ellipsoid portion of the photoreceptor cells. The presence of high concentrations of compartmentalized CK isoenzymes within photoreceptor cells (≈30 enzyme units/mg) as well as the relatively high concentration of total creatine in these cells (≈10–15 mM) indicates an important physiological function for CK and phosphocreatine in the energy transduction of vision.**

In skeletal and heart muscle, some of the creatine kinase (CK; ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) has been found to be localized in an isoenzyme-specific way at different subcellular structures or in different compartments—e.g., in the myofibrillar M-band, on the sarcoplasmic reticulum, on the plasma membrane, and within the mitochondria (for review, see refs. 1–3). It is thought that at these different locations, CK represents an ATP-regenerating system functionally coupled to microcompartments that require high local ATP/ADP ratios and immediate replenishment and supply of ATP—e.g., the actin-activated Mg^{2+}-ATPase of myosin, the sarcoplasmic Ca^{2+}-ATPase, or the sarcolemmal Na^+/K^+-ATPase, all being sites of high energy consumption. The soluble part of the muscle isozyme (MM-CK) and of the mitochondrial isoform (MiMi-CK) are thought to be functionally coupled to the ATP-producing systems of glycolysis and oxidative phosphorylation, respectively (3). The microcompartmentation of CK at these sites allows rapid transfer of metabolically active ATP into phosphocreatine, which represents a storage as well as a transport form of energy-rich phosphate. Thus, the sites of high energy requirement and energy production are thought to communicate by a phosphocreatine-shuttle (1–5)—a model that has gained increasing support also by recent experiments using novel phosphorus NMR techniques with high resolution at a faster time scale (6). In order to find out whether the phosphocreatine shuttle is a general metabolic feature of tissues with immediate high energy requirements, as has been postulated for muscle (7), electric organ of *Torpedo marmorata* (8), and sperm cells from sea urchin (9), chicken, and man (10), we studied the distribution of CK in chicken retina. A study by Dontsov *et al.* (11) indicated the presence of CK activity in retina and a possible association of this enzyme with the photoreceptor cells. We report here on the identification and localization of BB-CK and MiMi-CK in the photoreceptor cells of the retina. These results present yet another example for the presence of compartmentalized CK isoenzymes in tissues with high energy requirement. Parts of this work have been presented in abstract form (12).

MATERIALS AND METHODS

Analytical Procedures. Retinas from freshly killed adult chickens were dissected free of pigment epithelium and homogenized by Polytron mixer (Kinematika, Kriens, Switzerland) in 4–5 vol of phosphate-buffered saline (PBS) (0.15 M NaCl/10 mM sodium phosphate, pH 7.0) containing 3 mM 2-mercaptoethanol either with or without 1% Triton X-100. After incubation for 30 min on ice and short sonification, aliquots of the supernatants from centrifugation at 30,000 × *g* for 15 min were used. CK activity was measured by pH stat as described in detail (5). One enzyme unit corresponds to 1 μmol of phosphocreatine transphosphorylated per min/mg of retinal protein. Total creatine was determined colorimetrically with α-naphthol reagent by the method of Eggleton *et al.* (13). Protein was determined by the method of Lowry *et al.* (14) with bovine serum albumin (Pierce) as a standard.

Electrophoresis of tissue extracts (1–5 μl) on cellulose polyacetate strips (Gelman Sepraphore III) was performed under native conditions in 0.06 M Veronal buffer (pH 8.6) containing 1 mM 2-mercaptoethanol for 3 hr at 250 V. Staining for CK activity was achieved by the coupled enzyme agar overlay–gel technique (15). Staining due to myokinase activity was inhibited by 0.3 mM diadenosine pentaphosphate (16).

Immunoblotting of tissue extracts after separation of the proteins on 10% polyacrylamide/$NaDodSO_4$ gels (17) and electrophoretic transfer of the protein bands onto nitrocellulose (18) was performed as described (10) by using specific rabbit anti-chicken B- and Mi-CK antibodies (10) and fluorescein isothiocyanate-conjugated or peroxidase-labeled goat anti-rabbit IgG (Cappel Dynatech, Kloten, Switzerland).

Immunofluorescence in Frozen Sections of Retina. After a circular incision had been made into the front of adult chicken eyes and the vitreous humor had been removed, eyecups were prefixed in 2% paraformaldehyde and 0.1% glutaraldehyde in PBS (pH 7.2) for 30 min at 0°C. Retinas were removed and fixed for an additional 2 hr in the same fixative.

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Abbreviations: CK, creatine kinase; M-CK, B-CK, and Mi-CK, muscle, brain, and mitochondrial subunits of CK, respectively; MM-CK, BB-CK, MB-CK, and MiMi-CK, isoforms found in tissues.

Small pieces (2 × 3 mm) of retinas were immersed into 10% gelatin in PBS at 37°C; after solidification on ice, gelatin–retina blocks (5 × 5 mm) were cut and hardened with fixative for 30 min at 0°C. After the gelatin-stabilized retina blocks were washed with PBS, immersed in PBS containing 2.3 M sucrose overnight at 4°C (19), and frozen in liquid Freon 22, sections of 0.5-μm thickness were cut at −100°C with an ultracut E microtome (Reichert-Jung, Vienna) fitted with a cryokit FC4. Sections were picked up with a drop of PBS containing 2.3 M sucrose suspended in a steel-wire loop and mounted on coverslips. To wash out the sucrose, coverslips with the sections were placed several times onto droplets of 0.1 M glycine in PBS, and the sections were incubated for 30 min at 20°C with rabbit anti-chicken B-CK, anti-Mi-CK, or control IgG diluted 1:100 to 1:200 with PBS containing 10% normal goat serum to give a final concentration of approximately 1–5 μg/ml of specific antibodies. The sections were washed with PBS and stained for 30 min at 20°C by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel Dynatech) diluted 1:100. The sections were washed in PBS and mounted in 50% glycerol buffered with 0.1 M glycine/NaOH at pH 9.0. For epifluorescence microscopy, a Zeiss standard model 18 microscope equipped with a Planapo 63× oil immersion objective and a type III RS fluorescein/rhodamine filter set-up was used, and pictures were taken on Ilford HP-5 film.

RESULTS

The specific CK activity in extracts from adult chicken retina was 6.5 ± 1 μmol of phosphocreatine per min/mg of extracted retina protein. Addition to the extraction medium of 1% Triton X-100 increased the extractability of total CK activity by ≈20%, indicating that a fraction of CK is associated with particular subcellular structures as has been shown for mitochondria (4), for myofibrils (19), and with postsynaptic, acetylcholine receptor-rich membranes of *Torpedo marmorata* electrocytes (8, 20). Total creatine concentration in adult chicken retina was determined to be ≈3 mM ± 0.5 mM. As shown below, most of the CK is concentrated in the photoreceptor cell layer, representing ≈20% of the total retina mass; thus, the actual specific CK activity and the total creatine content within photoreceptor cells may be as high as 30 μmol of phosphocreatine per min/mg and 15 mM, respectively. These values approximate those found in skeletal muscle (3) and indicate that photoreceptor function, like muscle contraction, sperm motility (9, 10), and electrocyte discharge (21), depends on phosphocreatine hydrolysis.

Zymograms obtained by cellulose polyacetate electrophoresis of retina extracts followed by staining for CK activity indicate the presence of two CK isoenzymes, BB-CK and MiMi-CK (Fig. 1*A*, lane 3). BB-CK comigrated with CK extracted from chicken brain (Fig. 1*A*, lane 1) but not with CK from skeletal muscle (Fig. 1*A*, lane 2). Therefore, as had been shown for brain and spermatozoa (10), BB-CK and MiMi-CK are both present also in retina. Furthermore, significant adenylate kinase activity, which can be inhibited by diadenosine pentaphosphate, was also found in retina (not shown). Thus, photoreceptor cells seem to possess a high ATP-regeneration potential.

CK isoprotein subunits in adult retina extract were also identified by immunoblotting with subunit-specific rabbit anti-chicken B- and Mi-CK antibodies (Fig. 1*B*, lanes 4 and 6). B-CK and Mi-CK subunits were migrating on NaDodSO$_4$/polyacrylamide gels as M_r 43,000 and M_r 42,000 proteins, respectively (10). Simultaneous incubation of electrophoretically transferred protein bands from brain (Fig. 1*B*, lane 7) and retina (Fig. 1*B*, lane 8) with both antibodies led in both cases to a doublet of bands (arrowheads) corresponding to B-CK and Mi-CK, respectively. No staining was observed

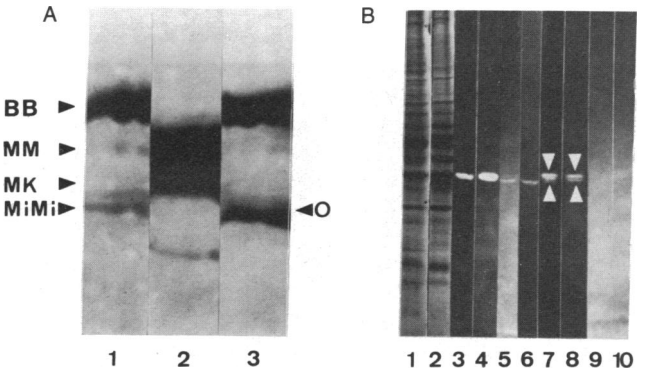

FIG. 1. Identification of CK isoenzymes in retina extracts. (*A*) Zymograms. Extracts of chicken brain (lane 1), skeletal muscle (lane 2), and retina (lane 3) after electrophoresis on cellulose polyacetate strips and staining for CK activity. Myokinase activity (MK) also present in retina (not shown) was abolished by diadenosine pentaphosphate (lane 3). O, origin of sample application. (*B*) Immunoblots. NaDodSO$_4$/10% polyacrylamide gel electrophoresis of total homogenates from chicken brain (lanes 1, 3, 5, 7, and 9) and retina (lanes 2, 4, 6, 8, and 10) stained for protein with Coomassie blue (1, 2) or incubated after transfer to nitrocellulose paper with the following antibodies: anti-B-CK (lanes 3 and 4), anti-Mi-CK (lanes 5 and 6), both anti-B- and anti-Mi-CK (lanes 7 and 8), or preimmune IgG (lanes 9 and 10). This was followed by staining with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (3–10). Note the presence of both B-CK and Mi-CK isoenzyme subunits with apparent M_r values of 43,000 and 42,000, respectively (ref. 10), resulting in antibody-stained doublet bands of brain and retina samples (7, 8).

with the preimmune control IgG (Fig. 1*B*, lanes 9 and 10) and anti-M-CK antibodies (not shown). As judged from Fig. 1, the amount of BB-CK in retina seems to be 5–10 times higher as compared with MiMi-CK.

After staining of cryosections from adult chicken retinas by the indirect immunofluorescence technique with anti-B-CK antibodies, strong labeling of the photoreceptor cell layer was observed (Fig. 2 *a* and *c*). Significant staining also was seen in the afferent nerve fibers (not shown), the horizontal cells (Fig. 2*a*, arrow), and the presynaptic region subjacent to the outer nuclear layer. Stratified staining presumably due to ordered arrangement of synapses was observed in the inner plexiform layer (Fig. 2*a*, arrowheads).

At higher magnification the strong staining with anti-B-CK antibody was localized within the ellipsoid and myoid portions of the inner photoreceptor segments (Fig. 2*c*) and at the periphery of the outer segments. In addition, weaker and diffuse staining of the outer nuclear layer, sparing the nuclei of the photoreceptor cells (Fig. 2*c*), was seen.

Incubation of retina cryosections with anti-Mi-CK antibodies followed by fluorescein isothiocyanate-conjugated second antibody yielded staining of the ellipsoid portion of the inner segments of rods and cones known to be rich in mitochondria (Fig. 2*e*). Staining with preimmune IgG (Fig. 2*g*), with antibodies preabsorbed with the purified antigens (BB-CK or MiMi-CK), or with anti-M-CK antibody (not shown) was negligible. It is noteworthy that very similar results were obtained with retinas embedded in Epon–Araldite or in paraffin. Use of affinity-purified antibodies yielded identical results. The structural integrity and the resolution of subcellular details of cryosections through retina obtained by the gelatin-support technique described here (Fig. 2) were by far superior to the latter two techniques, although the cryosectioning technique is rather difficult to perform, especially with retina. In addition, sections through different regions of the retina gave all very similar results.

Three types of oil droplets were found in chicken retina (Fig. 2 *g* and *h*). Type 1 droplets appeared dark by phase contrast, orange in bright field, and strongly fluorescent

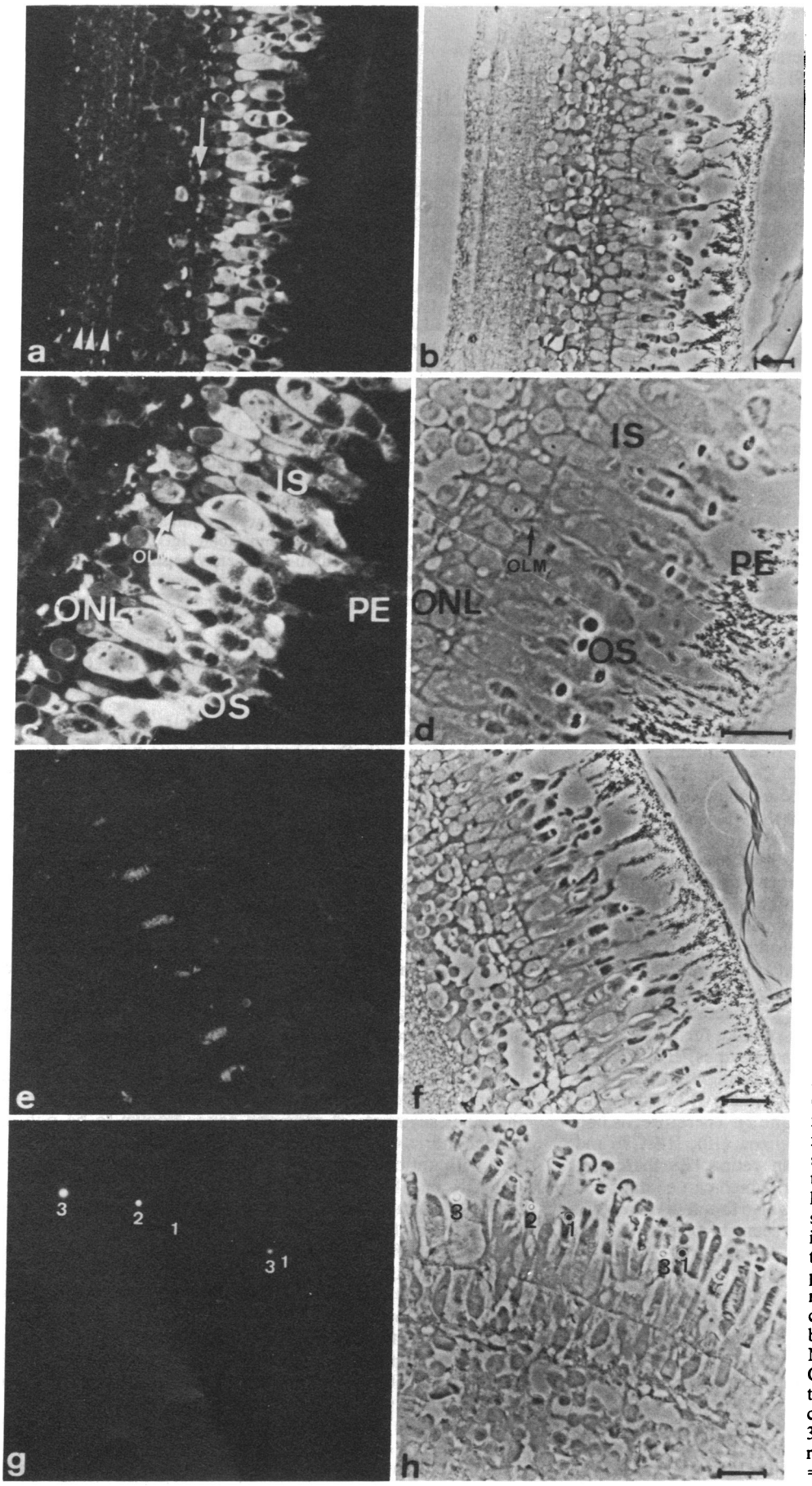

FIG. 2. Localization of CK isoenzymes in retina by indirect immunofluorescence staining of thin frozen sections. Cryosections of adult chicken retina stained by anti-chicken B-CK (*a*, *b*, *c*, and *d*), anti-Mi-CK (*e* and *f*) antibodies or with control IgG (*g* and *h*), followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. (*a* and *c*) Note the strong staining of photoreceptor cells by anti-B-CK antibody. Arrowheads and long arrows in *a* indicate stratified staining by anti-B-CK antibody of the inner plexiform layer and the horizontal cells, respectively. (*c* and *d*) PE, pigmented epithelium; OS, outer segment; IS, inner segment of photoreceptors; OLM, outer limiting membrane; ONL, outer nuclear layer. (*e*) Note the distinct staining by anti-Mi-CK antibody of mitochondria within the inner segments of photoreceptor cells. (*g* and *h*) The numbers 1, 2, and 3 indicate the three different types of retinyl ester-bearing oil droplets. (Bar = 25 μm; ×240–440.)

under rhodamine-specific illumination. Type 2 droplets appeared light in phase, yellowish in bright field, and strongly fluorescent under fluorescein isothiocyanate-specific illumination. Type 3 droplets appeared light in phase, colorless in bright field, and less fluorescent compared with type 2 droplets under fluorescein isothiocyanate-specific illumination. These different characteristics may be related to different precursors of photopigments stored in separate retinyl ester-bearing oil droplets (22) or to the color filter function attributed to them (23).

In conclusion, in adult chicken retina, both BB-CK and MiMi-CK are accumulated in separate subcellular compartments, mainly in the photosensitive rod and cone photoreceptor cells. Thus, the CK isoenzymes are present in those cells of the retina that depend on a high and sudden supply of energy.

DISCUSSION

BB-CK in retina, presumably being a mostly soluble enzyme although a fraction of it may be associated with membranes (1, 8, 10), is located in the inner and outer segments of photoreceptor cells, whereas MiMi-CK is strictly located within the mitochondria (4, 10) in the ellipsoid portion of the inner segment. As has been suggested for muscle (1–3) and spermatozoa (9, 10), a phosphocreatine shuttle between mitochondria in the ellipsoid portion and the different sites of high energy demand distributed over all of the photoreceptor cells may also be postulated for retina. According to such a model, CK would form microcompartments with ATP-requiring systems that are located within the photoreceptor cells at different subcellular sites that communicate via a phosphocreatine shuttle with the sites of energy production (for details, see ref. 3).

A wide spectrum of ATP-requiring reactions has been identified in connection with the visual transduction process. CK may be involved in the maintenance of high ATP/ADP ratios as well as in the immediate regeneration of ATP in a number of reactions where ATP is consumed—e.g., the ATP-dependent regeneration and phosphorylation of the rhodopsin photopigment by rhodopsin kinase (for review, see ref. 24), the ATP-dependent Na^+/K^+-ATPase of the photoreceptor cell membrane (25, 26), the ATP-requiring synthesis and transport of photoreceptor membrane components from inner to outer segments (27), the ATP-driven actomyosin-dependent retraction of photoreceptor cells, as reported for fish photoreceptor cells (28), and the ATP-dependent phosphotidylinositol/inositol trisphosphate-signaling pathway recently described in retina by Brown and Rubin (29) and Vandenberg and Montal (30) or, last but not least, the ATP requiring neurotransmitter synthesis and storage or even cyclic GMP metabolism. In darkness, where the unstimulated photoreceptors are continuously depolarized, virtually 90% of the energy is used to pump out Na^+ while, under light stimulation, the photoreceptor is hyperpolarized by blocking Na^+ entry and the release of the neurotransmitter is stopped. It is important to note that photoreceptor cells must deliver energy to ensure the functioning of their signaling system that not only has to respond rapidly to external signals (light) but also can maintain responsiveness over an extended period of time. This may be the very reason for the presence of high concentrations of CK in photoreceptor cells that seem to depend on a phosphocreatine-driven energy metabolism (31). Therefore, it makes sense to postulate also in retina a phosphocreatine shuttle system similar to that described for muscle and spermatozoa, where metabolic channeling of high-energy phosphate between mitochondria and sites of the ATP utilization is mediated by phosphocreatine (9, 10).

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