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# Brain-type creatine kinase in photoreceptor cell outer segments: role of a phosphocreatine circuit in outer segment energy metabolism and phototransduction

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## SUMMARY

**Different isoforms of creatine kinase, an important enzyme of vertebrate energy metabolism, were localized in bovine photoreceptor cells, with particular emphasis on the identification and quantification of the brain-type isoform within the outer segment compartment. Using immunofluorescence and immunoelectron microscopy, brain-type creatine kinase was shown to be present in bovine photoreceptor cell outer and inner segments. The presence of this isoenzyme in rod outer segments was additionally confirmed by immunoblotting and immunolabeling of isolated rod outer segments. The content of creatine kinase in rod outer segments was quantified by measuring creatine kinase activity after membrane disruption with detergent. The ATP regeneration potential provided by the creatine kinase in isolated, washed bovine rod outer segments was 1.2 ± (0.4) i.u. · $mg^{-1}$ 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 rod outer segments, and high enough to regenerate the entire ATP pool of rod outer segments within the time span of a photic cycle.**

**A mitochondrial creatine kinase isoenzyme was located within the ellipsoid portions of bovine rod and cone inner segments by immunofluorescence microscopy and, using immunogold staining, was specifically localized in the mitochondria clustered within bovine rod and cone inner segments. These results suggest that vertebrate photoreceptor cells contain a functional phosphocreatine circuit. Outer segment creatine kinase may play an important role in phototransduction by providing energy for the visual cycle, maintaining high local ATP/ADP ratios and consuming protons produced by enzymes located in the outer segment.**

Key words: creatine kinase, energy metabolism, retina, photoreceptor cells, rod outer segments

## INTRODUCTION

Recent advances in understanding the physicochemical and biochemical basis of phototransduction have led to a quite detailed picture of the visual cascade in vertebrate photoreceptor cells. Photic stimulation of the photoreceptor cell outer segment leads to hydrolysis of cyclic GMP and elicits the electrical response of vertebrate photoreceptor cell by closing the cGMP-gated cation channel (Chabre and Deterre, 1989; Kaupp and Koch, 1992). One of the major energy-consuming processes occurring in photoreceptor cell outer segments during phototransduction is the regeneration of the hydrolysed cGMP (Goldberg et al., 1983; Ames et al., 1986) from ATP and GTP, mediated by enzymes present in the outer segment (Hall and Kühn, 1986; Sather and Detwiler, 1987). Additional ATP-requiring processes like the NADPH-dependent chromophore regeneration (Schnetkamp and Daemen, 1981) or light-dependent rhodopsin phosphorylation (Kühn, 1974; Wilden and Kühn, 1982) also occur in this subcellular compartment.

Despite the prevalence of these energy-consuming processes in outer segments, the origin of the ATP required to sustain these events remains unclear. ATP levels remain stable upon illumination of rod outer segments (ROS) (Robinson and Hagins, 1979; Biernbaum and Bownds, 1985b), suggesting the presence of a potent ATP-regenerating system in close proximity to the energy-requiring processes. Although some enzymes of the glycolytic pathway were recently found in bovine ROS preparations (Hsu and Molday, 1990, 1991; Lopez-Escalera et al., 1991), glycolysis is not thought to represent a major ATP-regenerating system in ROS (Lopez-Escalera et al., 1991). Accordingly, the levels of the glycolytic enzymes are generally low in outer segments as compared to inner segments or other retinal layers (Lowry et al., 1961; Berger et al., 1980).

Oxidative phosphorylation also cannot directly provide the ATP needed in the outer segments, since the mitochondria of the elongated polar vertebrate photoreceptor cells are located exclusively in the inner segments (Hughes et al., 1972), and the delivery of ATP from the inner to the outer segment is impaired by the short diffusion length of nucleotides (Yoshizaki et al., 1990). Though oxidative phosphorylation cannot be the direct source of ATP, photoreceptor cells contain most of the retinal mitochondrial activity and are functionally dependent upon oxidative phosphorylation (Buono and Sheffield, 1991). These observations imply that other ATP-regenerating systems, coupled to mitochondrial oxidative phosphorylation, must replenish ATP depleted by the various energy-consuming processes in ROS.

In cells such as muscle or spermatozoa (Wallimann et al., 1984; Tombes and Shapiro, 1985; Tombes et al., 1987) a 'phosphocreatine circuit' (Wallimann et al., 1989, 1992) is proposed to connect oxidative phosphorylation in the mitochondria to ATP consumption in the cytosol. The key enzyme in this circuit is creatine kinase (CK; EC 2.7.3.2), which catalyzes the reversible transfer of the phosphoryl group from phosphocreatine (PCr) to ADP, to generate ATP and creatine. The circuit connects spatially distant energy-producing and energy-consuming processes by replacing ATP and ADP with PCr and creatine as the diffusive metabolites. If a PCr circuit that is functionally linked to outer segment phototransduction events were to exist in photoreceptor cells, cytosolic CK must be present in the outer segments and mitochondrial CK (Mi-CK) in the mitochondria of inner segments. According to such a hypothesis, CK would be essential for the energetics of phototransduction by maintaining constant cytosolic ATP levels.

Two isoforms of CK, brain-type (B-CK) and mitochondrial CK (Mi-CK) were found in chicken retina (Wallimann et al., 1986b). Both of these CK isozymes are expressed at high levels but are distributed differentially within the inner segments of these cells. While Mi-CK is restricted to the mitochondria-rich ellipsoid portion, B-CK was localized in both the ellipsoid and myoid portions of chicken inner segments (Wallimann et al., 1986b). Although some data suggested that B-CK was present in outer segments (Dontsov et al., 1978; Schnetkamp and Daemen, 1981; Wallimann et al., 1986b; Wegmann et al., 1991), unambiguous evidence for the presence of the cytosolic CK isoform in photoreceptor cell outer segments, and quantitative analysis thereof, was missing.

Thus we used immunological and biochemical methods to localize CK isoenzymes in photorecptor cells, with special emphasis on whether B-CK can be unambiguously identified and quantified in the outer segment compartment of bovine photoreceptor cells. Since outer segments are structurally more clearly defined and can be better isolated in bovine retina, which contains predominantly rod-type photoreceptor cells, than in chicken retina, containing predominantly cone cells (Szél et al., 1986), bovine retina and ROS were chosen for detailed studies of CK isoenzyme distribution and content.

## MATERIALS AND METHODS

### Reagents and enzymes

ADP was obtained from Boehringer Mannheim (Rotkreuz, Switzerland); PCr was from Calbiochem (Luzern, Switzerland). The Pharmalytes™ pH 5-8 were purchased from Pharmacia (Uppsala, Sweden). Both the dye-reagent for the protein assay and the low molecular mass protein standard for SDS-PAGE were from Bio-Rad (Glattbrugg, Switzerland). Other reagents used were of analytical grade quality. Emulphogene BC-720 (polyoxyethylene 10-tridecyl ether) and rabbit B-CK were from Sigma (Buchs, Switzerland).

### Assays

CK activity was determined by directly measuring the transphosphorylation rate of PCr in the reverse CK reaction ($MgADP^- + PCr^{2-} + H^+ \xrightarrow{CK} MgATP^{2-} + Cr$; Watts, 1973) by the pH-stat method using the described assay mixture (Wallimann et al., 1984), supplemented with 0.1% (v/v) detergent (Emulphogene BC-720) and, when indicated, with 20.5% sucrose (w/v). One enzyme unit (i.u.) corresponds to 1 μmol of PCr transphosphorylated per minute at 25°C at pH 7.0. Protein concentrations were determined as described (Bradford, 1976), using Bio-Rad reagent with bovine serum albumin (Pierce, Oud-Beijerland, The Netherlands) as a standard. Protein concentration values were corrected, according to the specifications of the manufacturer (Bio-Rad), by a factor of 2.1. Rhodopsin concentrations were determined essentially according to Wilden and Kühn (1982) from the difference in absorbance at 500 nm before and after bleaching, in the presence of 1% Emulphogene BC-720 and 50 mM hydroxylamine at pH 7.2.

### Electrophoretic analysis and immunoblotting

Retina and ROS extracts were separated on 10% polyacrylamide-SDS gels, and proteins were electrophoretically transferred onto nitrocellulose as described (Wegmann et al., 1991). Non-specific binding sites on the nitrocellulose blots were blocked with blocking buffer consisting of 10% horse serum and 0.1% BSA in PBS (phosphate buffered saline: 137 mM NaCl, 2.6 mM KCl, 6.4 mM $Na_2HPO_4$, 1.4 mM $KH_2PO_4$, pH 7.4). B-CK was specifically detected with affinity-purified rabbit anti-chicken B-CK IgG at 1:250, followed by peroxidase-conjugated goat anti-rabbit IgG diluted either at 1:5,000 (Calbiochem, Luzern, Switzerland) or at 1:2,500 (Nordic, Lausanne, Switzerland) using 4-chloro-1-naphthol as substrate (Wegmann et al., 1991). ROS proteins were further characterized by two-dimensional gel electrophoresis; in the first dimension, isoelectric focussing between pH 5 and 8, a pH range known to resolve the heterogeneous B-CK forms of chicken particularly well (Quest et al., 1990), was used.

### Immunofluorescence labeling of cryosectioned retina

After removal of the vitreous body, the eye-cups obtained from cattle were prefixed with 3% paraformaldehyde/0.1% glutaraldehyde in PBS for 30 minutes on ice. In some experiments the pigment epithelium and the cornea were removed from the other retinal layers before the retina was fixed for an additional 2 hours in the same fixative. Small pieces (2 mm × 3 mm) of retina were immersed in 10% gelatine in PBS at 37°C; after solidification on ice, the retina-containing gelatine blocks were cut (5 mm × 5 mm) and hardened with fixative for 30 minutes on ice, washed with PBS, immersed in 2.3 M sucrose in PBS overnight at 4°C and frozen in liquid Freon 22 or liquid nitrogen. Sections of 0.5 μm were cut at −100°C with an Ultracut-E ultra-microtome (Reichert-Jung, Vienna, Austria), picked up with a drop of 2.3 M sucrose in PBS and mounted on coverslips for immunofluorescence. Cov-

erslips with sections were placed on droplets of 0.05 M glycine in PBS and blocked with 0.2% gelatine, 1% BSA in PBS for 30 minutes at room temperature. Following the blocking step, the specimens were incubated for 1 hour with the primary antibody or preimmune sera diluted at 1:100 in PBS containing 1% BSA and then washed for 3× 5 minutes with PBS. Subsequently they were incubated for at least l hour with FITC-coupled second antibody diluted 1:200 in PBS containing 1% BSA, before being washed again for 3× 5 minutes with PBS and mounted in 50% glycerol buffered with 0.1 M glycine-NaOH at pH 9.0. Preparations were examined with a Zeiss model 18 epifluorescence microscope and photographed on Ilford HP-5 film. B-CK was localized using previously characterized anti-chicken-B-CK sera and affinity-purified anti-chicken B-CK IgG (Wallimann et al., 1986a,b; Quest et al., 1990; Wegmann et al., 1991). Outer segments were identified with a polyclonal rabbit antibody, raised against frog ROS proteins (Rungger-Brändle and Leuenberger, 1987). The Mi-CK staining was performed using a polyclonal antibody raised against the Mi-CK isoform expressed in chicken brain (Wyss et al., 1990). Rabbit preimmune sera were used for control stainings.

## Preparation of ROS

Cattle eyes were obtained from the local slaughter house immediately after the animals' death and kept on ice in the dark. ROS were prepared in dim red light at 4°C following a procedure described previously (Wilden and Kühn, 1982) with some modifications. In short, 30 eyes were opened by a circular incision at the front to remove the vitreous humor. Batches of 15 retinas were collected in a 40 ml plastic Sorvall tube with 15 ml of Ringer solution (130 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM EDTA, 5 mM glucose, 1 mM DTT and 15 mM $NaH_2PO_4$, pH 7.2) and the ROS detached by vigorous agitation on a Vortex shaker for 30 seconds at maximum speed. The homogenate was filtered through 100 μm mesh Scrynel fabric and the resulting filtrate applied to a step gradient containing 10 ml of 32% and 12 ml of 20.5% sucrose in Ringer. ROS accumulated at the gradient interphase during centrifugation for 30 minutes at 10,000 ***g***, were removed with a syringe, diluted twofold in 20.5% sucrose in Ringer and sedimented for 10 minutes at 5,000 ***g***. The pellets were gently resuspended in 20.5% sucrose in Ringer solution and sedimented as before. Pellets were resuspended and sedimented up to four times. Alternatively, the sedimentation steps were omitted and instead the ROS from the step-gradient interphase were diluted twice with Ringer and washed on a second and third step gradient of 32% and 20.5% sucrose in Ringer. These ROS preparations are referred to as 'washed ROS'.

## Immunofluorescence labeling of isolated ROS

ROS were stabilized using an 8% paraformaldehyde/0.6% glutaraldehyde fixative mixture in Ringer or PBS for 4 hours. The fixative solution was supplemented with 20.5% sucrose. Prefixed ROS suspensions were diluted 1:10 with Ringer, stored overnight at 4ºC and sedimented for 10 minutes at 5,000 ***g***. The resulting pellet was resuspended in 2.3 M sucrose and stored at 4°C. A drop of fixed ROS suspension was briefly dried at room temperature on a glass slide. To remove sucrose and to quench remaining fixative, the samples were washed for 3× 5 minutes with PBS containing 50 mM glycine. ROS were then permeabilized for 10 minutes with 0.1% Triton X-100 in PBS and subsequently washed for 3× 5 minutes with PBS. After non-specific binding sites had been saturated by treatment with blocking buffer for 1 hour, ROS were immunofluorescently labeled using the conditions described for cryosectioned retinas. For immunofluorescence staining of bovine ROS a commercially available rabbit anti-human B-CK antibody (Cambridge Medical Diagnostics, Billerica, MA, USA) was either used directly or after affinity purification by the method of Olmsted (1981). The same results were obtained when rabbit anti-chicken-B-CK serum (Wallimann et al., 1986a,b; Wegmann et al., 1991) was used. In control experiments anti-B-CK immune sera were preincubated with an excess of purified rabbit B-CK (at 1.5 $mg \cdot ml^{-1)}$ for 30 minutes on ice and centrifugated prior to incubation with the specimen.

## Cryosections of isolated ROS

ROS were centrifuged and resuspended in PBS containing 2.3 M sucrose and frozen in liquid nitrogen. Cryosections were made as described above and mounted on Formvar-coated copper grids. Sucrose was washed out by incubating the grids for 2× 10 minutes in PBS-glycine. Then the cryosections were contrasted for 2× 10 minutes in 4% neutral uranyl acetate, followed by an incubation for 1 minute in distilled water and for 2 minutes in 2% acidic uranyl acetate before being stabilized by 1.3% methyl-cellulose and air dried. Preparations were examined with a Jeol 100C electron microscope at 100 kV and photographed on Agfa (Scienta) 6.5 cm × 9 cm plates.

## Immunogold labeling on plastic sections

### High-pressure freezing, freeze-substitution and low temperature embedding

Bovine retina were cut into small pieces (1 mm × 1 mm) and put between two aluminium platelets (each 0.5 mm thick, 3 mm in diameter with cavities of 0.3 mm) after soaking the sample with 1-hexadecene (Studer et al., 1989). This sandwich was immediately frozen with liquid nitrogen at a pressure of 2000-2500 bar (1 bar = $10^5$ Pa) in the high-pressure freezing machine, HPM 010, from Balzers, Liechtenstein (Müller and Moor, 1984) and stored in liquid nitrogen. Freeze-substitution was performed with 100% ethanol as substitution medium in a freeze-substitution device (FSU 010 from Balzers, Liechtenstein) at 176 K and at 213 K for 10 hours at each step. Infiltration of the tissue with the methacrylate resin, Lowicryl HM20 (Lowi GmbH, Waldkraiburg, Germany), was done successively in mixtures with ethanol (1:1 (v/v) for 30 minutes, 2:1 (v/v) for 2 hours and 100% resin for 20 hours) at 213 K.

For polymerization, UV light with 360 nm wavelength was used for 1 hour at 213 K, for 26 hours at 223 K and at room temperature for 2–3 days. Ultrathin sections of 50 nm were cut with the Ultracut (Reichert-Jung, Vienna, Austria).

### Immunogold labeling and silver enhancement

The non-specific binding sites of sections from retina embedded in Lowicryl HM 20 resin (see above) were blocked for 3 hours with blocking solution 1 (PBS with 0.5% bovine serum albumin, 0.2% gelatine, 1% newborn goat serum and 2 mM sodium azide). The primary antibodies were diluted in blocking solution 1, and sections were incubated with the diluted antibodies for 1–2 hours at room temperature. After several washing steps with blocking solution 1, a biotinylated goat anti-rabbit antibody (diluted 1:500 in blocking solution 1) was applied for 90 minutes at room temperature. After carefully washing, the biotin goat anti-rabbit antibody was followed by AuroProbeOne streptavidin, a 1 nm gold probe conjugated to streptavidin. The gold probe was diluted 1:50 in blocking solution 2 (blocking solution 1 with omission of newborn goat serum) prior to application for 75 minutes. Sections were washed several times with blocking solution 2 followed by PBS and postfixed with a mixture of 3% paraformaldehyde and 0.1% glutaraldehyde. After several washing steps with PBS and finally ultrapure water, reagent A (enhancer) and reagent B (initiator) were mixed 1:1 and immediately used for 5 minutes at 295 K (IntenSE M silver enhancement kit from Janssen Pharmaceutica, Beerse, Belgium). Signal amplification was stopped by washing with ultrapure water. Sections were poststained with aqueous

uranyl acetate and Reynolds' lead citrate (Reynolds, 1963) and examined in a Jeol 100C at an acceleration voltage of 100 kV.

For immunolabeling on plastic sections, the purified IgG fraction of polyclonal rabbit antiserum against B-CK from chicken brain was obtained by using a Protein A-Sepharose CL-4B affinity column from Pharmacia (Uppsala, Sweden). The antibodies were eluted from the column with a 100 mM citrate/sodium citrate buffer, pH 3.0, and immediately neutralized with 2 M Tris-HCl, pH 9.0. For storage, eluted IgG fractions were concentrated with a YM 10 membrane in an Amicon ultrafiltration unit (Amicon, MA, USA) and kept frozen. The highest signal in B-CK labeling of outer segments could be reached by using cryofixed, freeze-substituted and low-temperature-embedded Lowicryl HM20 sections, compared with other techniques tested. The signal could also be improved by the application of small gold particles, 6 nm (not shown) or 1 nm colloidal gold that had to be enhanced with silver.

### Immunogold labeling on cryosections

Bovine retina was fixed with a mixture of 3% paraformaldehyde and 0.5% glutaraldehyde at 277 K. Fixed tissue was vitrified with 2.3 M sucrose in PBS for 2 hours. Small pieces were mounted on stubs and frozen in liquid nitrogen. Sectioning was performed in a freezing chamber of the Ultracut from Reichert-Jung (Vienna, Austria) at 173 K. Sections (100 nm) were collected in TBS (Tris-buffered saline, pH 7.4) supplemented with 20 mM glycine, incubated for 30 minutes with blocking solution (TBS containing 0.5% bovine serum albumin and 0.2% gelatine), and subsequently incubated for 1 hour with specific antibodies diluted in blocking solution. After washing in TBS, goat anti-rabbit antibody coupled to 10 nm gold particles (from Janssen Pharmaceutica, Beerse, Belgium) was diluted 1:20 in blocking solution and was applied for 30 minutes. Several washing steps with TBS and ultrapure water followed before the sections were treated with neutral uranyl acetate for 5–10 minutes on ice. The last staining and stabilization step was performed with a mixture of 0.1 ml 3% aqueous uranyl acetate and 0.9 ml 3% methyl-cellulose (1500 poise; 1 P = $10^{-1}$ Pa·s) for 10 minutes on ice. Polyclonal rabbit antibody directed against chicken cardiac Mi-CK was affinity purified as described (Schlegel et al., 1988). Anti-ROS antibody was the same as described earlier.

## RESULTS

### Localization of CK isoenzymes in bovine retina by immunofluorescence microscopy

Vertebrate photoreceptor cells are specialized neurons consisting of an outer segment, connected by a thin stalk to the inner segment, the nucleus and a synaptic termination. Due to the polar array of these cellular compartments, photoreceptor (PR) cells show a stratified appearance under the light microscope (Fig. 1). Since we were particularly interested in the localization of brain-type CK in the outer segment compartment, this compartment was unambiguously identified not only by its close proximity to the retinal pigment epithelium but also by immunofluorescence labeling (Fig. 1c,d and g,h) with an antibody raised against frog ROS proteins (Rungger-Brändle and Leuenberger, 1987).

Indirect immunostaining of cryosectioned bovine retina with isoenzyme specific anti-B-CK and anti-Mi-CK antibodies revealed an accumulation of CK isoenzymes within the photoreceptor cell layer. B-CK was present in outer segments (compare anti-B-CK staining in Fig. 1a,b with anti-ROS staining in Fig. 1c,d). The shapes of individual bovine outer segments could be traced by the anti-B-CK immunofluorescence signal in compact bovine retina (Fig. 1a,b, arrowheads). This staining pattern was also found in anti-B-CK stainings of bovine retina where, in order to improve the fixation of the photoreceptor layer, the pigment epithelium and adjacent layers had been removed during fixation (not shown). B-CK immunoreactivity was also pronounced in inner segments, particularly in cone cells (Fig. 1a), supporting earlier data with chicken retina (Wallimann et al., 1986b). Furthermore, while the cell bodies of the photoreceptor cells in the outer nuclear layer showed very low levels of anti-B-CK immunoreactivity, some B-CK was localized between the photoreceptor cell bodies in the outer nuclear layer (Fig. 1a). In control stainings essentially no labeling was observed (not shown).

Anti-Mi-CK serum specifically labeled the inner segments of the photoreceptor layer where staining was most intense in the ellipsoid portion of cone cells (Fig. 1e,f, arrow). Staining was also visible in inner segments of the predominant rod cells (Fig. 1e,f, double arrowhead), which can be easily distinguished from cone cell inner segments by their different morphology. Again, anti-ROS staining of a parallel section is shown to identify and illustrate the outer segment layer (Fig. 1g,h).

### Immunogold labeling of B-CK and Mi-CK in photoreceptor cells of bovine retina

To localize B-CK in the outer segments of the photoreceptor cells more precisely, immunogold localization was performed with bovine retina (Fig. 2). Outer segments were identified by the typical appearance of densely staggered disk membranes, as well as with a specific marker antibody (Fig. 2c). B-CK was clearly localized within the central areas of the outer segments (Fig. 2a) in addition to its localization in the inner segments (Fig. 2b). No obvious difference was observed in labeling intensity of outer segments derived from either rod or cone cells. Controls showed no labeling (Fig. 2d).

The immunogold staining pattern obtained with anti-Mi-CK antibody differed markedly from that with anti-B-CK antibody. The mitochondrial isoform of creatine kinase was detected in the mitochondria, which are exclusively located in the inner segments of photoreceptor cells; essentially no labeling of outer segments was found. Anti-Mi-CK staining was most intense in the ellipsoid portions of cone cells, where mitochondria are densely clustered (Fig. 3a). In micrographs at higher magnification it is shown that Mi-CK is localized at cristae membranes and in the space between the inner and outer membrane (Fig. 3b). Almost no gold particles were observed in control stainings, using rabbit preimmune serum (Fig. 3c).

### Characterization of B-CK in isolated bovine ROS

The previous immunolocalization results strongly suggest that B-CK is located in bovine ROS. To characterize ROS B-CK, bovine ROS were isolated. The average yield for ROS from 11 ROS preparations was 13.1 (± 0.8)%, as was judged by comparison of the rhodopsin content of isolated ROS with that of total retina. The latter was measured to be approximately 1.2 (± 0.2) mg rhodopsin per retina.

Analysis of bovine ROS pellets by SDS-PAGE revealed

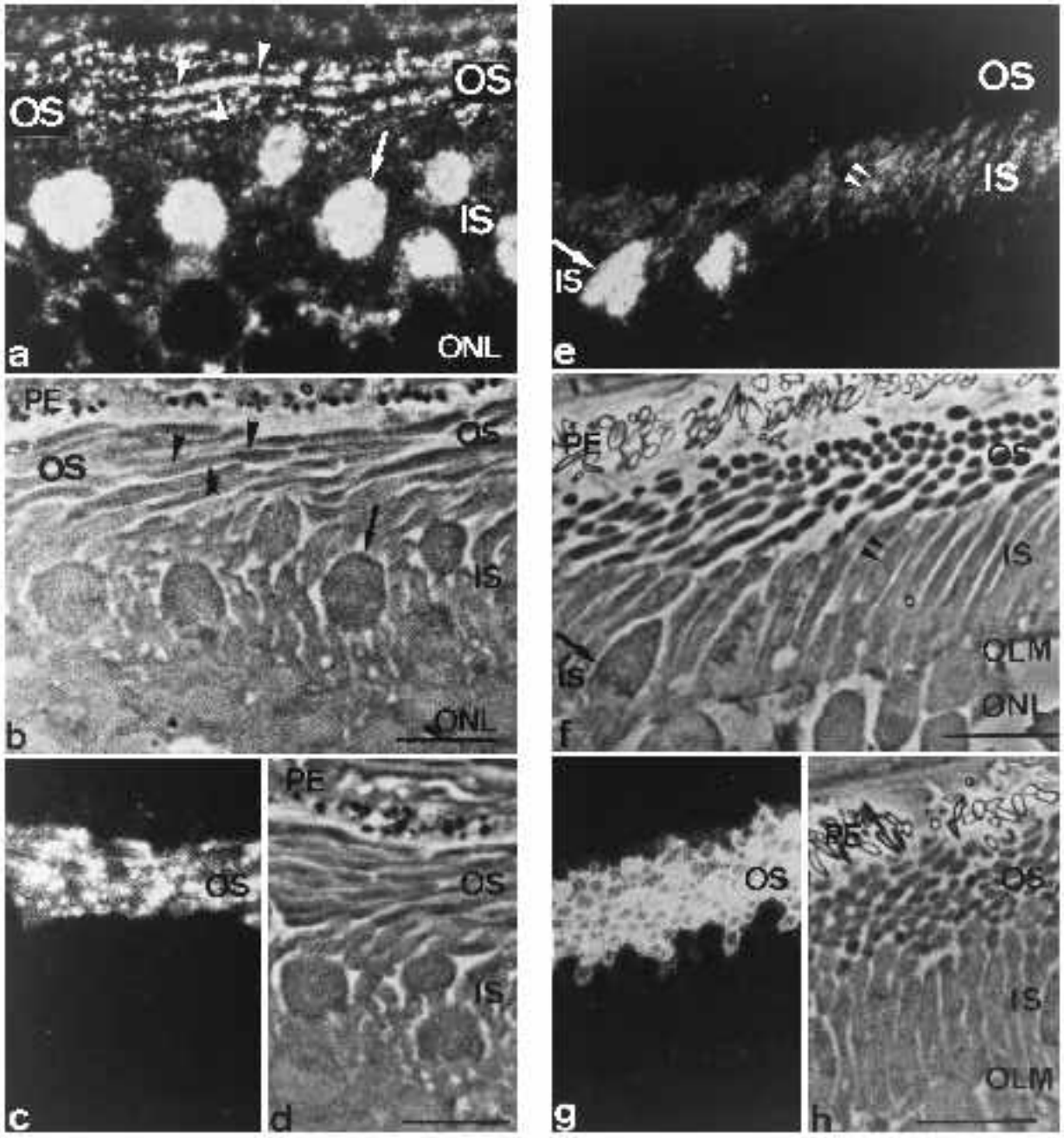


**Fig. 1.** Localization of B-CK and Mi-CK in the photoreceptor cell layer of intact bovine retina by indirect immunofluorescence staining of cryosections. Anti-chicken B-CK (a,b) and anti-Mi-CK labelings (e,f) are shown together with their respective parallel sections (c,d and g,h), stained with an antibody raised against frog ROS proteins to unambiguously identify the outer segment layer (OS). (b,d,f and h) represent the corresponding phase-contrast images of (a,c,e and g). Note the strong in situ labeling in inner, as well as the specific staining of outer, segments with the anti-B-CK antibody (a). By following the anti-B-CK immunofluorescence signal, both the location and outline of individual ROS could be traced within the outer segment layer as indicated by arrowheads in (a) and (b). B-CK immunoreactivity was also found in cone inner segments (indicated by arrows in a,b). Mi-CK is specifically localized both within inner segments of cones (single arrow in e,f) and rod cells (double arrowheads in e,f). ONL, outer nuclear layer; OLM, outer limiting membrane; IS, inner segments; OS, outer segments; PE, pigment epithelium. Bars, 10 μm.

typical ROS protein compositions (Fig. 4, lane 5) with prominent bands in the $M_r$ region of 35,000 to 40,000 (opsin, transducin) as well as in the $M_r$ region of 95,000 (phosphodiesterase, PDE). A protein band with approximate $M_r$ values of 43,000 to 45,000 was also apparent in SDS-gels (marked by an asterisk). A single protein band of this $M_r$ was strongly labeled by the affinity-purified anti-B-CK antibody when bovine retina extracts (Fig. 4, lanes 2,7) or extracts from ROS (Fig. 4, lanes 5,10) were analysed by western blotting. Extensive development of the western blots led to the appearance of a faint band with a slightly lower apparent $M_r$ as compared to B-CK, which was appar-

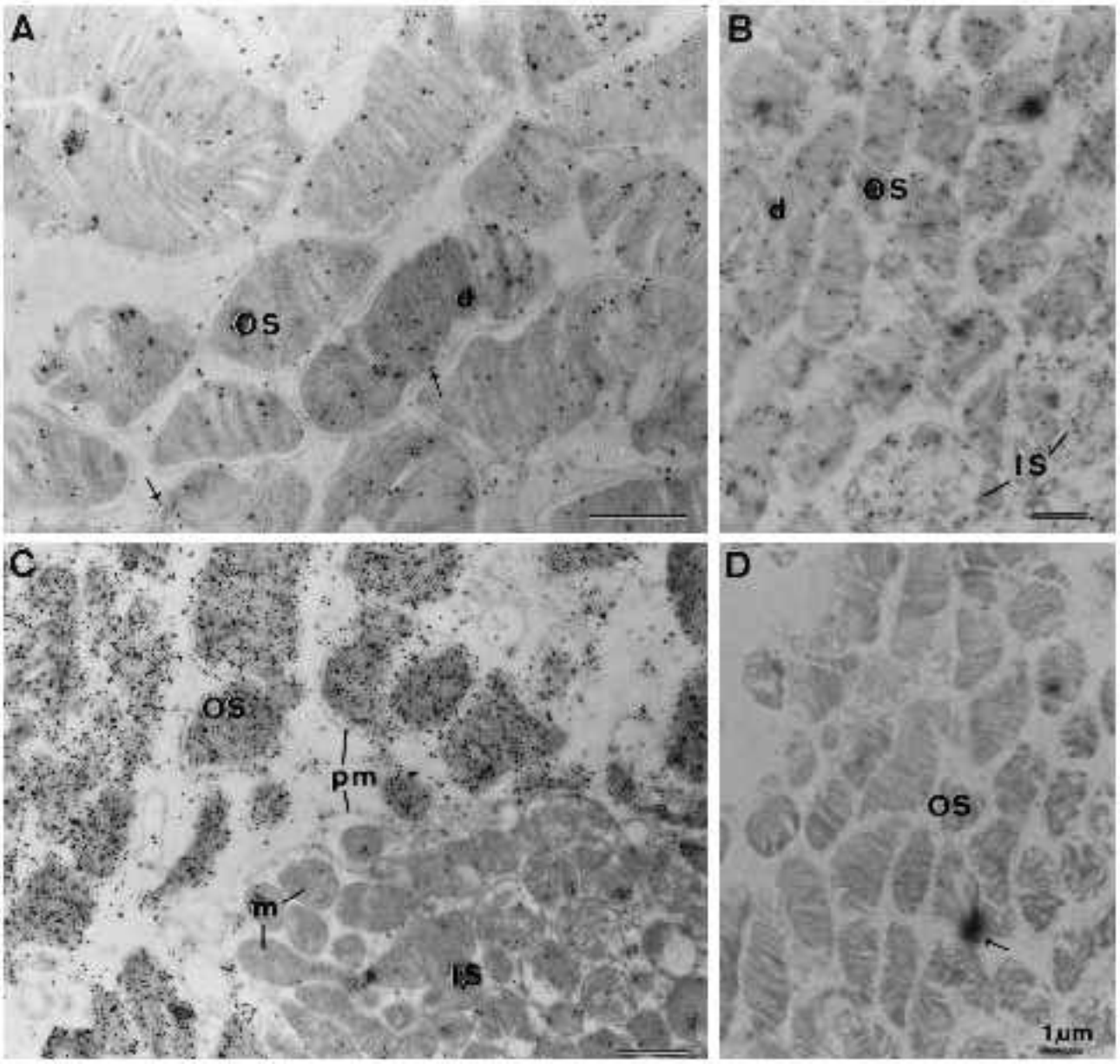


**Fig. 2.** Immunogold localization of B-CK in bovine photoreceptor cells. Immunolabeling of high-pressure-frozen, freeze-substituted and Lowicryl HM20 low-temperature-embedded bovine retina. Labeling by rabbit anti-chicken B-CK IgG at higher (A) and lower magnification (B) is shown together with outer-segment-specific staining (C) by antibodies raised against ROS proteins. (A–D) show the outer segments and parts of the inner segments of the photoreceptor cells labeled with 1 nm gold particles that were enlarged to 15–40 nm by the precipitation of metallic silver. B-CK is found in the interior of the outer segments (OS) with membranous disks (d) and sometimes was also found near the plasma membrane surrounding the outer segments (arrows). B-CK is also localized in inner segments (B). Using an antibody against ROS proteins (C), outer segments of rods and cones are densely labeled by the silver-enhanced colloidal gold particles whereas inner segments are mostly devoid of them. A part of the mitochondria-rich (m) portion of the inner segment of a cone cell is shown here. Outer and inner segments are surrounded by the plasma membrane (pm) of the photoreceptor cells. In the absence of primary antibody (D), there is no background staining. The applied enhancement time of 5 minutes at 295 K was short enough to avoid extensive formation of silver precipitates by self-nucleation (arrow, D), but long enough to yield a highly amplified signal (from 1 nm to 40 nm) as seen in A, B and C. OS, outer segments; IS, inner segments. Bars, 1 µm.

ently due to a slight crossreactivity of the antibody with Mi-CK, also present in total retina (Fig. 4, lane 7). However, this band (Mi-CK) was not detectable in ROS extracts (Fig. 4, lane 10). This slight crossreactivity was only detectable on blots but not by immunocytochemistry. In addition, CK isoenzyme electrophoresis (Ishida et al., 1991) was used to confirm that B-CK is the only CK isoenzyme present in ROS (not shown).

Assuming that the Coomassie blue staining at $M_r$ 43,000 to 45,000 was due only to B-CK and allowing for correction of staining intensity due to the twofold lower $M_r$ of B-CK compared to PDE, these results suggest that B-CK and

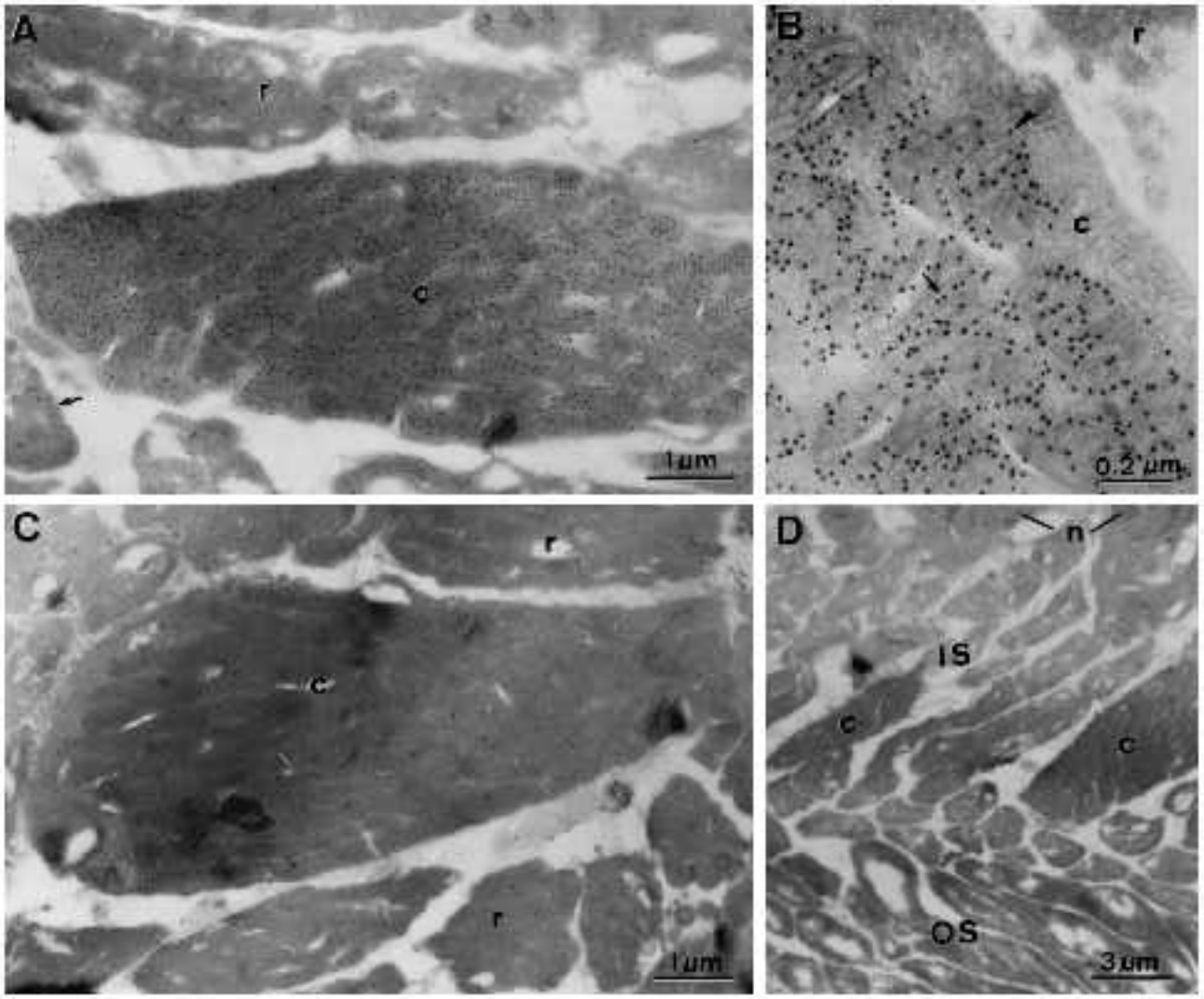


**Fig. 3.** Immunogold localization of Mi-CK in bovine photoreceptor cell inner segments. Cryosections of bovine retina labeled with affinity-purified anti-Mi-CK antibody (A, B and D) or preimmune serum (C) and 10 nm colloidal gold particles. (A) The densely packed mitochondria in the ellipsoid portion of the inner segments of cone cells (c) are very strongly labeled for Mi-CK. Mitochondria contain numerous regularly ordered cristae membranes, which appear bright on a dark matrix background. The same specific labeling but with a lower intensity takes place in the fewer mitochondria of rod inner segments (A, small arrow). Rod mitochondria are more difficult to distinguish at this magnification. Bar, 1 µm. (B) Higher magnification of a Mi-CK labeling as shown in (A). A part of the inner segment of a cone cell containing numerous mitochondria is represented. Gold particles are located in the intracristae space alongside the cristae membranes (small arrow), or between the inner and outer mitochondrial membrane (arrowhead). Because of the negative membrane contrast, all the membranes display a bright contrast whereas the intermembrane space displays a dark one. Bar, 0.2 µm. (C) Very few gold particles can be observed in the mitochondria-rich cone cell or in the rod cell when preimmune serum was used as primary antibody. Bar, 1 µm. (D) Survey of the Mi-CK-labeling shown in A illustrating outer and inner segments of the photoreceptor cells. Two cone cells (c) are lying between the thinner rod cells. Also parts of two nuclei (n) of the photoreceptor cells are represented. Bar, 3 µm. OS, outer segments; IS, inner segments; r, rod cell.

PDE are present in roughly similar quantities in ROS. In frog ROS, PDE α and β together represent roughly 2% of total ROS protein (Hamm and Bownds, 1986).

Finally, analysis of bovine ROS by 2D gel electrophoresis followed by immunoblotting revealed the existence of only one major CK subunit spot with an acidic satellite spot. The 2D pattern of bovine ROS B-CK (Fig. 4C) is similar to that of rat B-CK. The acidic satellite spots of rat and chicken B-CK are due to phosphorylation (Mahadevan et al., 1984; Quest et al., 1990).

## Localization and quantification of B-CK in isolated ROS

Isolated washed bovine ROS showed strong immunofluorescence signals when labeled with affinity-purified anti-B-CK antibodies (Fig. 5a,b). Staining intensity was drastically reduced when the same antibody was first saturated with an excess of purified rabbit B-CK (Fig. 5c,d). The isolated ROS were structurally well preserved even after extensive washing procedures (see Materials and Methods), as shown by electron microscopy (Fig. 5e).

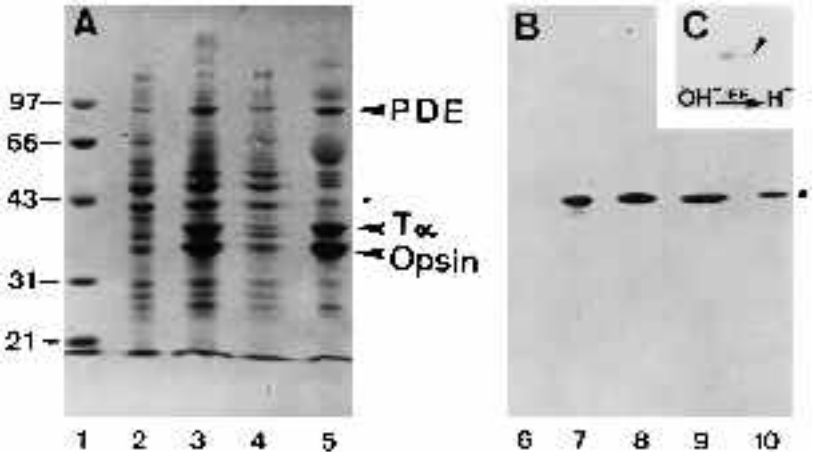


**Fig. 4.** Characterization of bovine ROS preparations by analysis on one- and two-dimensional PAGE. (A) shows a 10% polyacrylamide-SDS gel after Coomassie-Blue staining: lane 1, $M_r$ standard proteins indicated ($\times 10^{-3}$); lane 2, bovine retina homogenate after vortexing (containing 0.2 i.u. of CK activity); lane 3, ROS from sucrose gradient interphase (0.2 i.u. of CK activity); the same sample as in lane 3 was centrifuged (5 minutes, 15,000 ***g***) and supernatant (lane 4) and pellet (lane 5) were analyzed separately. Note the predominance of typical ROS proteins in lane 5: phosphodiesterase (PDE), transducin (Tα) and opsin. Oligomers of opsin give diffuse bands at higher apparent $M_r$ (65,000 and 100,000). (B) shows the samples from (A) after electrophoretic transfer onto nitrocellulose and subsequent detection of B-CK with affinity-purified anti-B-CK IgG diluted at 1:250, followed by horseradish peroxidase (HRP)-conjugated second antibodies. The position of the B-CK band is indicated by an asterisk. (C) shows the region of interest on a nitrocellulose blot after 2D gel electrophoresis of the same sample shown in lane 5, followed by blotting and subsequent detection of B-CK with affinity-purified anti-B-CK IgG. Note the resolution of a single main B-CK monomer species in bovine ROS B-CK and an additional minor satellite species indicated by the arrowhead.

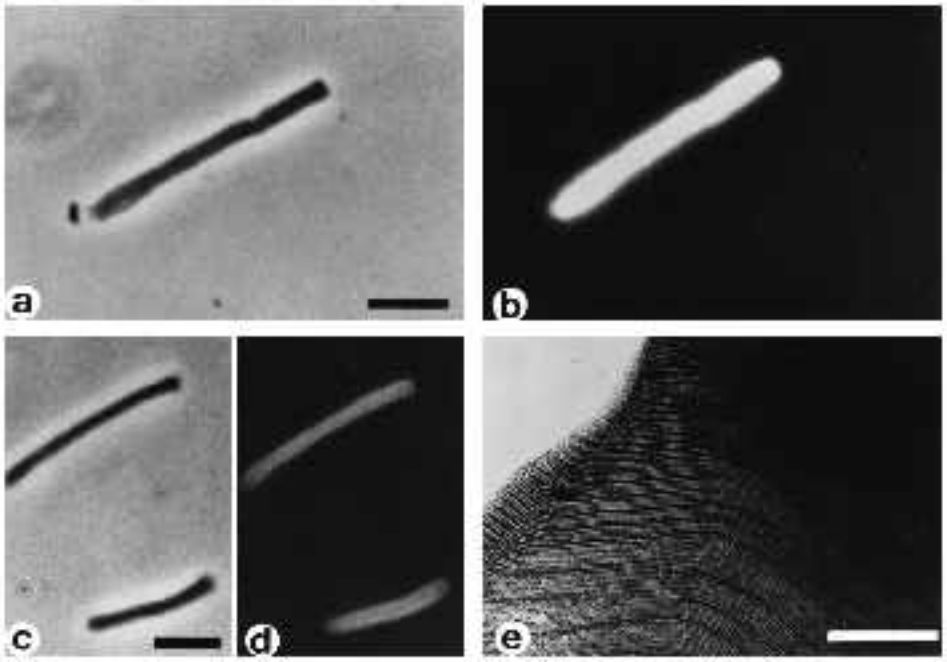


**Fig. 5.** Immunofluorescence staining for B-CK of isolated bovine ROS. Washed bovine ROS (see Materials and Methods) stained by indirect immunofluorescence for B-CK (a,b); control after pre-incubation of affinity-purified anti-B-CK antibody with an excess of rabbit B-CK (c,d); both followed by FITC-conjugated second antibody. Phase-contrast (a,c); fluorescence (b,d). Ultrathin cryosection of bovine ROS observed by electron microscopy (e). Note the good structural preservation of bovine ROS disks and plasma membrane. Bar, 5 μm (a-d) or 150 nm (e).

This good structural preservation of bovine ROS also allowed the quantification of the enzymatic CK activity in such ROS preparations. Data of a typical isolation procedure are shown in Table 1. Even after several cycles of sedimentation and washing of the ROS in Ringer solution containing 20.5% sucrose (not shown) or after multiple sucrose step gradients, the values of CK activity in ROS, measured by the pH-stat method, ranged between 1 and 2 i.u. per mg rhodopsin (Table 1). Additional cycles of washing did not result in a decrease in this value (not shown). In 11 bovine ROS preparations, the average CK activity of washed ROS was 1.2 (± 0.4) i.u. per mg rhodopsin, with a maximal value of 2.3 i.u. per mg rhodopsin. Thus, washed bovine ROS contained significant amounts of CK activity.

## Detergent-dependent release of B-CK activity from within isolated ROS

To prove unequivocally that the B-CK activities measured for isolated washed ROS originated from within ROS, the detergent dependency of the release of B-CK from ROS was tested. In a pH-stat assay mix supplemented with 20.5% sucrose to prevent ROS lysis, only negligible CK activity was measured in the absence of detergent (Fig. 6b). However, CK activity was immediately measurable when washed ROS were lysed by adding them to an assay mixture containing 0.1% detergent (Fig. 6a). When CK activity in ROS was measured for 8 minutes in the absence of detergent (Fig. 6b), a slight increase in CK activity was observed.

**Table 1. Isolation of bovine ROS; data from a typical experiment**

| | Protein* (mg) | Rhodopsin (mg) | Specific CK activity (i.u. · mg rhodopsin$^{-1}$) |
|---|---|---|---|
| Crude extract | 978 | 16.4 | 139 |
| Filtrate | 307 | 8.7 | 98 |
| ROS interphase (1st gradient) | 10.6 | 5.0 | 3.6 |
| ROS interphase (2nd gradient) | 5.2 | 4.8 | 1.1 |
| ROS interphase (3rd gradient) (washed ROS) | 2.8 | 2.6 | 1.2 |

*The absolute protein value of total ROS protein may be slightly underestimated, since rhodopsin, which constitutes approximately 70% of total ROS protein (Hamm and Bownds, 1986), might show a disproportionally low binding of the dye used in the protein determination assay.

The detergent-independent release of CK activity, comprising only 12% of the final activity measured after detergent addition to the same assay mixture, is likely to be caused by ROS lysis through shear forces present in the continuously stirred pH-stat assay mixture. When ROS were analysed in the normal hypotonic assay mix (as Fig. 6b, but without sucrose or detergent), CK activity increased during 8 minutes to about 39% of the final value determined in the presence of detergent (not shown). Neither detergent nor sucrose had any detectable effect on the enzymatic activity of purified B-CK itself (not shown). Only slight baseline activities could be attributed to the presence of sucrose. Additional proton consumption was entirely dependent on the presence of PCr and ROS in the assay mix. These experiments confirmed that the CK activities determined for ROS were due to CK located within the ROS compartment, and not due to activity fortuitously bound to ROS surfaces.

## DISCUSSION

### B-CK in ROS: presence of a PCr circuit in photoreceptor cells

In sperm, a PCr circuit is required for the transport of high energy phosphates between the mitochondria near the sperm head and the flagellar axoneme (Tombes and Shapiro, 1985; Tombes et al., 1987), which can be up to 40 μm long. The highly polar photoreceptor cells show an analogous organization. Many energy-requiring processes of phototransduction occur in the outer segments of these cells, while oxidative phosphorylation, which is crucial for photoreceptor cell function (Buono and Sheffield, 1991), is restricted to the inner segments (Hughes et al., 1972). The distance between the inner segments and distal end of the outer segment ranges between 20 and 50 μm, depending on the species.

The goal of this work was to provide evidence for the existence of a functional PCr circuit in photoreceptor cells, which would require the presence of a cytosolic CK isoform in the outer segment and Mi-CK in the inner segment mitochondria. Mi-CK was indeed located exclusively in the inner segments of bovine rod and cone cells, using immunofluorescence staining (Fig. 1e) and immunogold labeling (Fig. 3a) with anti-Mi-CK antibodies. Anti-Mi-CK staining was most prominent in the ellipsoid portion of the cone cells where mitochondria are densely clustered. Immunofluorescence and immunogold stainings of bovine retina demonstrated that B-CK is located in outer segments in addition to its presence in inner segments (Figs 1a and 2). The CK isoform present in ROS was confirmed to be B-CK by immunoblotting (Fig. 4A,B), 2D gel analysis (Fig. 4C) and native CK isoform electrophoresis (not shown). Washed bovine ROS were shown to contain 1-2 i.u. CK activity per mg of rhodopsin. In addition, immunolocalization studies of extensively washed ROS (Fig. 5), together with the detergent-dependent release of CK activity from ROS (Fig. 6), confirmed that B-CK is located within the ROS and is not adventitiously bound to the outer membrane surface. Bovine ROS isolated by a different method (Molday and Molday, 1987; a kind gift from Dr R.S. Molday, Vancouver) were also shown to contain B-CK by western blot analysis (not shown). Thus all prerequisites for a functional PCr circuit operating as a system for energy shuttling from inner to outer segments in bovine photoreceptor cells are fulfilled.

In the remainder of this Discussion we provide corroborating evidence that a PCr circuit would be sufficient to meet the energy requirements of photoreceptor cells, and

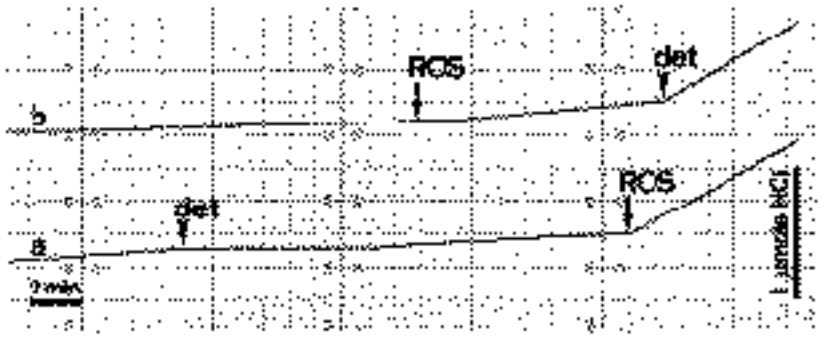


**Fig. 6.** Detergent-dependent release of B-CK activity from washed bovine ROS. CK activity was directly monitored by measuring proton consumption of the CK in the direction of ATP synthesis. Original pH-stat tracings demonstrate that CK activity found in extensively washed bovine ROS preparations was released in a detergent-dependent manner from within the ROS. Both tracings started with a negligible baseline activity. Addition of detergent (indicated by arrowheads) led to a slight, transient pH effect (trace **a**) but did not change the baseline activity or influence the activity. Addition of ROS (55 μg rhodopsin in each experiment) is indicated by arrows. When detergent was present in the assay prior to the addition of washed ROS, an immediate increase in proton consumption was detected (trace **a**). In the absence of detergent only negligible activity was measured in an 8 minute time period, but proton consumption increased immediately when detergent was added (trace **b**; see also Results). The measured proton consumptions were fully dependent on the presence of PCr in the assay mix (not shown), demonstrating that only CK can account for the activities measured.

that the amount of B-CK present in outer segments is sufficient to regenerate the ATP required in the outer segments within the time frame of a phototransduction event.

Roughly equimolar pools of ATP and GTP exist in ROS (Robinson and Hagins, 1979; Berger et al., 1980), interconnected by two ROS proteins, nucleoside diphosphate kinase (NDPK) and guanylate kinase (GK) (Berger et al., 1980; Hall and Kühn, 1986). In isolated outer segments, ATP levels are generally buffered more effectively than the GTP levels (Biernbaum and Bownds, 1985b). Upon bright illumination GTP levels fall far below ATP levels (Biernbaum and Bownds, 1985b), suggesting that ATP may serve as a precursor of GTP. Furthermore, GTP cannot replace ATP in fueling the pentose phosphate pathway, which recycles the NADPH necessary for chromophore reduction (Schnetkamp and Daemen, 1981). Thus, ATP is apparently the main energy source in outer segments.

The source of outer segment ATP has been unclear. Although the ATP turnover is high, ATP levels in ROS remain constant upon photic stimulation of photoreceptors (Robinson and Hagins, 1979; Biernbaum and Bownds, 1985b; Ames et al., 1986). The stability of ATP levels upon illumination is indicative of the presence of a powerful ATP-replenishing system in this subcellular compartment. As mentioned in the Introduction, oxidative phosphorylation cannot directly provide ATP in the outer segment, since outer segments do not contain mitochondria, and glycolysis is not thought to be an important ATP-regenerating system in ROS. On the other hand, it was reported that light flashes bleaching 1% of outer segment rhodopsin cause the enzymatic loss of millimolar amounts of PCr with the appearence of equivalent amounts of inorganic phosphate (Yoshikami and Hagins, 1985). Considering the different enzymes of GTP and ATP metabolism that have been identified in ROS, we suggest that the resynthesis of cGMP via GTP, catalysed by guanylate cyclase (GC) within the outer segment compartment (Kaupp and Koch, 1992), might occur at the final expense of PCr according to the following reaction scheme:

(1) $cGMP^- \xrightarrow{PDE} GMP^{2-} + H^+$
(2) $Mg{\cdot}GTP^{2-} \xrightarrow{GC} cGMP^- + Mg{\cdot}PP_i^{2-} + H^+$
(3) $2\ Mg{\cdot}ADP^- + 2\ PCr^{2-} + 2\ H^+ \xrightarrow{CK} 2\ Mg{\cdot}ATP^{2-} + 2\ Cr$
(4) $Mg^{2+} + GMP^{2-} + Mg{\cdot}ATP^{2-} \xrightarrow{GK} Mg{\cdot}ADP^- + Mg{\cdot}GDP^-$
(5) $Mg{\cdot}ATP^{2-} + Mg{\cdot}GDP^- \xrightarrow{NDPK} Mg{\cdot}ADP^- + Mg{\cdot}GTP^{2-}$

$(\sum 1\text{-}5): Mg^{2+} + 2\ PCr^{2-} \xrightarrow{PDE,GC,CK,GK,NDPK} Mg{\cdot}PP_i^{2-} + 2\ Cr$

According to this scheme, PCr/Cr diffuse between inner and outer segments instead of cGMP and GTP, as was proposed earlier (Berger et al., 1980). The molecules PCr and Cr diffuse more readily than GTP and cGMP, because they are smaller in size, less negatively charged and, unlike cGMP (Kaupp and Koch, 1992), do not bind to membranes. Thus, we propose that a PCr circuit maintains the energy flux between the inner segments and the outer segments.

## Calculation of the amount of B-CK in ROS and ATP-regeneration potential in ROS

The average B-CK activity released from within washed bovine ROS upon treatment with detergent was 1.2 (± 0.4) i.u./mg of rhodopsin (maximal value 2.3 i.u./mg rhodopsin). These values represent 'lower-limit estimates', since some CK is always lost from bovine ROS due to leakage during the isolation and subsequent washing steps (H. Kühn, personal communication). Additionally, CK activity, expressed in i.u., is defined at 25°C. In vertebrate retina, at about 37°C, the enzymatic activity is expected to be 2.5-fold higher. Assuming the $M_r$ of rhodopsin to be 41,000 (Wilden and Kühn, 1982), the average and maximal values for ROS CK activity correspond to turnover rates of 46 and 87 PCr or ATP molecules $\cdot$ $min^{-1}$ per rhodopsin molecule, respectively. For the above reasons, this is a very conservative estimate of the ATP-regeneration potential provided by bovine ROS B-CK.

ATP concentrations in vertebrate ROS are generally believed to be between 0.2 mM and 1 mM (Berger et al., 1980; Schnetkamp and Daemen, 1981; Biernbaum and Bownds, 1985a), although values as high as 3 mM (Robinson and Hagins, 1979) have been reported. Rhodopsin concentrations ([Rh]) in ROS are in the range from 2.4 to 6 mM (Ames et al., 1986; Daemen, 1973; Lopez-Escalera et al., 1991; Liebman and Entine, 1968). Thus, it follows that the average (maximal) CK activity measured in ROS is capable of regenerating the entire outer segment ATP pool within 1.6 (0.9) seconds (assuming 3 mM [ATP] and 2.4 mM [Rh] in ROS) or 110 (60) milliseconds (assuming 0.2 mM [ATP] and 6 mM [Rh] in ROS). Assuming intermediate values for the ATP and rhodopsin concentrations (1 and 3 mM, respectively), the above ATP turnover time would be between 230 and 440 milliseconds. In any case, these values are within or even well below the time scale of photon-dependent ROS plasma membrane hyperpolarization and photocurrent recovery (Pugh and Cobbs, 1986; Chabre and Vuong, 1992).

The average (maximal) CK activity measured at 25°C in ROS translates into an ATP regeneration potential in this compartment between 1.9 and 4.6 (3.5 and 8.7) mM $ATP \cdot s^{-1}$ for rhodopsin concentrations of 2.4 or 6 mM [Rh], respectively. On the other hand, cGMP hydrolysis, a major energy-consuming reaction in ROS (Ames et al., 1986), requires 30 μM ATP equivalents $\cdot$ $s^{-1}$ in the dark. Upon light stimulation this value increases by a factor of 4.5 (Goldberg et al., 1983; Ames et al., 1986; Kaupp and Koch, 1992). Thus, even the 'lower-limit estimate' of CK activity measured in bovine ROS provides more than a tenfold excess in ATP-regeneration potential over ATP consumption due to cGMP hydrolysis upon photic stimulation. Accordingly, sufficient B-CK is present in ROS to supply energy for additional light-dependent ATP consuming reactions such as rhodopsin phosphorylation (Wilden and Kühn, 1982), chromophore reduction by NADPH (Schnetkamp and Daemen, 1981) or phosphorylation of ROS proteins by protein kinases (Binder et al., 1989; Smith et al., 1991).

## Additional roles for ATP and CK in ROS and inner segments

ATP is not only utilized as an energy source to regenerate cGMP, which is hydrolyzed during phototransduction (Goldberg et al., 1983; Ames et al., 1986), but also seems to be a key regulatory factor influencing cGMP levels. ATP rapidly quenches light-induced PDE activity (Liebman and Pugh, 1980; Sitaramayya and Liebman, 1983). This effect is mediated by the 48 kDa protein, arrestin (Glitscher and

Rüppel, 1989), which binds to phosphorylated rhodopsin (Kühn et al., 1984), preventing the activation of transducin (Wilden et al., 1986), and thus also inhibiting the cGMP phosphodiesterase (Chabre and Deterre, 1989). This double role of ATP in phototransduction was confirmed by recording the light-sensitive current during intracellular dialysis of fully functional ROS preparations with defined nucleotide-containing solutions (Sather and Detwiler, 1987). Additionally, ATP and a non-hydrolyzable ATP analogue induced a tenfold increase in cGMP-binding to bleached ROS, indicating that cGMP hydrolysis might not be the only mechanism by which cytosolic cGMP levels can be reduced in ROS (Fesenko and Krapivinsky, 1986). Thus, due to its ability to regulate ATP levels, CK in ROS may also be involved in ATP-mediated regulatory control of phototransduction.

A further important function of CK in the outer segment might be to prevent the acidification of this compartment. Both enzymes involved in cyclic cGMP turnover within ROS, phosphodiesterase and guanylate cyclase (reactions (1) and (2), respectively), produce protons. Since the equivalent of two molecules of ATP are consumed in the resynthesis of one molecule of cGMP, the equivalent enzymatic activity of CK would simultaneously reutilize the protons produced by both these enzymes and provide the required energy equivalents for the resynthesis of cGMP (reaction (3)).

Other functions of the CK/PCr system (reviewed by Wallimann et al., 1989, 1992) likely to be relevant to photoreceptor function include the maintenance of low intracellular ADP concentrations; high ADP concentrations would inactivate ATPases. In addition, this system may provide appropriate local ATP/ADP ratios, which ensure the thermodynamic efficiency of ATP hydrolysis ($\Delta G$) at the sites of ATP consumption. For instance, the high B-CK content of inner segments (Fig. 1a) may be needed to generate and maintain appropriate ATP/ADP ratios required for $Na^+,K^+$-ATPase function. This ATP-dependent ion pump is present exclusively in the plasma membrane of inner segments (Stirling and Lee, 1980), where its activity represents the main energy-consuming reaction during the resting state of visual transduction (Chabre and Vuong, 1992). Interestingly, CK was shown to be functionally coupled to the $Na^+,K^+$-ATPase in *Torpedo* electrocytes (Blum et al., 1991).

In summary, 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. On the other hand, 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 subject to control. A possible regulatory mechanism affecting either of the above properties may be protein phosphorylation. Rat (Mahadevan et al., 1984), mouse (Chida et al., 1990) and chicken B-CK (Quest et al., 1990) are phosphoproteins. The heterogeneity of bovine ROS B-CK observed on 2D gels (Fig. 4c) was similar to that reported for B-CK from other species, suggesting that some of the B-CK in bovine ROS is also phosphorylated. For mouse and chicken B-CK, phosphorylation was shown to alter the $K_m$ of the enzyme for PCr (Quest et al., 1990; Chida et al., 1990). Alternatively, phosphorylation might regulate the distribution of B-CK between membrane and cytosol, as has been demonstrated for other proteins (Kühn et al., 1984; Mascarelli et al., 1989). In this respect, it is interesting to note that some so-called cytosolic, vertebrate CK isoenzymes have also been shown to associate with membranes and subcellular structures in vivo and in vitro (Wallimann et al., 1984, 1986a; Rossi et al., 1990; Rojo et al., 1991), and the tail-specific CK isoform from sea urchin sperm was recently shown to be a membrane-associated protein (Quest and Shapiro, 1991), anchored by an N-terminal myristoyl moiety (Quest et al., 1992).

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