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Creatine kinase isoenzymes in spermatozoa*

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Summary

Two isoforms of creatine kinase (CK, E.C. 2.7.3.2), the brain type BB-CK and the mitochondrial-bound MiMi-CK, as well as adenylate kinase (myokinase, E.C. 2.7.4.3) were identified in washed spermatozoa from chicken and man by cellulose polyacetate electrophoresis and immunoblots. BB-CK was localized by indirect immunofluorescence staining within the sperm tail but not in the head portion. MiMi-CK is confined to the midpiece region rich in mitochondria and has been localized directly by immunogold staining within the mitochondria. In contrast to chicken, seminal plasma from man was also found to contain considerable amounts of BB-CK. Total creatine content of spermatozoa (8–15 mM) and seminal plasma (3.8 ± 0.4 mM) as well as preliminary experiments with metabolic blockers indicate a dependence of sperm motility on CK and phosphoryl creatine (CP). The presence of two CK isoforms located in different 'compartments' of spermatozoa suggests a CP-shuttle in sperm similar to that described for cross-striated muscle.

Introduction

While considerable data on the presence of brain-type creatine kinase (BB-CK) in semen of man has already been documented and its multiglandular origin from the prostate and the seminal vesicle postulated (Kavanagh & Darby, 1983), little is known about the presence of this enzyme within spermatozoa. The only report on the presence of some CK activity in spermatozoa, incidentally from roosters, was published by Buckland (1970). Up to now, however, no information on the type of CK isoenzymes or their localization has been available.

As for muscle, spermatozoan motility is dependent on ATP as the direct source of energy (Bishop, 1962), a fact elegantly demonstrated by Lindemann & Gibbons (1975). Thus the presence of creatine kinase as an ATP regenerating system within spermatozoa would be a definite physiological advantage for sperm motility. In muscle, some of the CK is found to be localized in an isoenzyme-specific way in different compartments, e.g. mitochondria, sarcoplasmic reticulum, plasma membrane and the myofibrillar M-band (Turner *et al.*, 1973; Wallimann *et al.*, 1977, 1978, 1983a, b; Saks *et al.*, 1978), thus forming functionally coupled microcompartments. Therefore, a phosphoryl creatine (CP) shuttle between ATP generating (glycolysis and mitochondrial oxidative phosphorylation) and ATP utilizing sites (myofibrillar actin-activated Mg^{2+}-ATPase, Na or K-ATPase and Ca^{2+}-ATPase), has been postulated (Wallimann, 1975; Saks *et al.*, 1978; Bessman & Geiger, 1981; Wallimann & Eppenberger, 1984). The present study was undertaken to demonstrate directly CK activity in isolated spermatozoa from chicken and man, to investigate the type of CK isoenzymes in total sperma and isolated spermatozoa and to find out about the localization of CK isoenzymes within spermatozoa.

Materials and methods

Isolation of spermatozoa

Fresh rooster sperma, kindly provided by D. Steiger from the Institut für Tierzucht ETH Zürich, was centrifuged at 1000–2000***g*** for 5–10 min. The supernatants were clarified of any residual debris by high speed centrifugation at 20 000***g*** for 10 min and the seminal plasma used directly for analysis or stored at −20° C. The first pellet containing spermatozoa was resuspended in isowash (0.25 M sucrose, 1–10 mM β-mercaptoethanol, 10 mM benzamidine, an inhibitor of trypsin-like proteases, and 2 mM EGTA at pH 7.4). After washing three times with isowash, the spermatozoa were resuspended with isowash to the original sperma volume and referred to as washed chicken spermatozoa.

Human sperma was obtained from healthy donors (according to the regulations of the Swiss–German Society for artificial insemination, Bulletin No. 2, September 1983). Immediately after liquefaction (30 min at 30–37° C) 1–2 ml portions of human semen were layered over 5 ml of prewarmed (37° C) Percoll solution (density, 1.05 g ml^{-1})

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and centrifuged at 1000*g* for 10 min at 20° C. Seminal plasma was clarified by centrifugation at 25 000*g* for 10 min and used directly for analysis. The spermatozoan pellet was resuspended in isowash to the original volume and the procedure repeated to yield Percoll–isowash washed human spermatozoa (Arcidiacono *et al.*, 1983).

Extraction of CK from washed sperms

CK was extracted from spermatozoa either by short sonication of washed spermatozoa in PBS or veronal buffer containing 0.1–0.5% Triton X-100 or by hypo-osmotic swelling of spermatozoa by resuspension in distilled water followed by extraction with 50 mM phosphate buffer at pH 8.5, a treatment known to extract mitochondrial CK (Jacobus & Lehninger, 1973).

Cellulose polyacetate electrophoresis of sperm extracts and subsequent staining for CK activity was performed according to published methods (Turner *et al.*, 1974). Omission of CP from and addition of diadenosine pentaphosphate (AP5A) (Cohen *et al.*, 1978) to the overlay gel were used to identify myokinase activity.

Immunoblots were performed according to the method of Towbin *et al.* (1979) as modified by Wallimann *et al.* (1982). Antibodies specific for native M-CK, B-CK and mitochondrial CK subunits from chicken characterized as described earlier (Walliman *et al.*, 1977, 1983a, b; Perriard *et al.*, 1978) were used. In addition, affinity purified anti-M and B-CK IgG were also used. These were specific for sodium dodecyl sulphate (SDS)-denatured CK isoenzyme subunits after cross-absorption with the heterologous CK isoenzyme. Anti-human B-CK IgG was purchased from Merck Laboratories, Darmstadt, West Germany, and anti-human mitochondrial CK was a gift from Dr B. Deus, Bad Homburg, West Germany (Blum *et al.*, 1983).

Immunofluorescence

For indirect immunofluorescence staining a diluted suspension of washed spermatozoa in isowash was smeared onto glass slides and dried at 20° C and 0.1 Torr for 10 min. This proved to be the most satisfactory method by which to attach the cells to the glass and prevent loss of spermatozoa during the steps that followed. To remove the sucrose, the spermatozoa were washed for 5–10 min by gentle immersion of the slides in an excess of phosphate-buffered saline (PBS). For subsequent permeabilization and prefixation of spermatozoa, slides were either dipped for 1 min into methanol at −20° C followed by three washes with PBS or were immersed for 1 min at 20° C in PBS containing 0.05–0.1% Triton X-100. After three washes with PBS the Triton-treated spermatozoa were prefixed with 3% paraformaldehyde in PBS for 25 min on ice quenched with 0.2 M glycine in PBS at pH 7.4 for 2 min on ice and finally washed in PBS. Spermatozoa permeabilized and fixed either by methanol or Triton–formaldehyde were incubated for 30 min at 20° C with anti-B-CK and anti-Mi-CK IgG at concentrations of 1–5 μg ml^{-1} of specific antibody diluted with PBS containing 1 mg ml^{-1} of bovine serum albumin (BSA) or incubated with preimmune IgG as controls. After three consecutive washes with PBS, FITC-conjugated goat anti-rabbit IgG (Cappel) at a dilution of 1:100 in PBS containing 1 mg ml^{-1} of BSA was applied to the same area on the slide. After 30 min at 20° C the slides were washed three times with PBS and mounted in 50% glycerol buffered with 0.1 M glycine–NaOH at pH 9.0. Preparations were examined with a Zeiss fluorescence microscope and photographed on Ilford HP-5 film.

Immunogold labelling of chicken spermatozoa

Freshly collected rooster sperma was centrifuged for 5 min at 3000*g*. The seminal plasma was decanted and the pelleted sperm carefully resuspended in a small volume of PBS. An equal volume of 2% glutaraldehyde and 2% paraformaldehyde in PBS was added immediately and the suspension left on ice overnight for fixation. After removing the fixative by washing with PBS, sperm were resuspended in a small volume of PBS to give a thick suspension. Then one volume of 10% (w v^{-1}) of melted gelatin in PBS rewarmed to 40–45° C was added to the sperm suspension at room temperature. The gelatin–sperm suspension was poured onto cool glass slides and left for solidification. Small gelatin–sperm squares (1–2 mm) were cut, transferred with PBS into test tubes and postfixed for at least 2 h on ice with 0.2% glutaraldehyde–2% paraformaldehyde in PBS. After washing out the fixative with excess PBS the fixed gelatin–sperm pieces were stored at 4° C or used directly. Before freezing the gelatin–sperm pieces were incubated for at least 10–12 h in 2.3 M saccharose in PBS for cryoprotection, frozen in liquified Freon or preferably, in liquid propane, mounted, and sections were cut using an Ultracut E microtome (Reichert-Jung, Vienna, Austria) fitted with a Cryokit FC 4 at 100 nm thickness.

Freshly cut ultrathin sections were transferred and placed for at least 5 min in 50 mM glycine in PBS for quenching of the fixative, transferred to 0.2% gelatin, 0.5% bovine serum albumin in PBS (referred to as PBG) and left on this solution for two 10 min periods. Sections were transferred onto 150 μl drops of rabbit anti-chicken MiMi-CK antiserum diluted 1:50 to 1:100 with PBG and incubated for 2 h at 20° C. For controls the same dilution of preimmune serum was used. After washing six times for 2 min each on 100 μl drops of PBG, sections were incubated on 150 μl drops of 1:100 diluted goat anti-rabbit IgG–gold complex (GarG with 10 nm colloidal gold; Janssen Pharmaceutica, Beerse, Belgium) and incubated for 1 h, washed seven times for 2 min each on drops of PBG, washed twice for 2 min on PBS–glycine and finally washed four times for 2 min each time with distilled water. Immediately thereafter the sections were stained for 5 min on 2% neutral uranyl acetate in 0.15 M sodium oxalate (pH adjusted with 5% NH_4OH to 7.4) and for an additional 5 min on 2% acidic uranyl acetate. Sections were mounted on grids in 1.3% Tylose MH 300 (Fluka, Switzerland) with platinum loops, air-dried and observed in a Jeol JEM 100C electron microscope at 100 kV. Photographs were taken on Agfa-Gevaert Scientia film.

Other procedures

Creatine kinase activity was measured at pH 7.0 with a Radiometer pH-stat as described in detail by Wallimann *et al.* (1984). The specific CK activity is expressed in μmol CP transphosphorylated min^{-1} ml^{-1} of protein at 25° C. Total creatine was determined colorimetrically with α-naphthol reagent according to the method of Eggleton *et al.* (1943) allowing in the perchloric acid extracts for complete

hydrolysis of phosphoryl creatine to creatine. Protein was determined according to the Biuret (Gornall *et al.*, 1949) or Lowry method (Lowry *et al.*, 1951). Polyacrylamide gel electrophoresis (PAGE) in the presence of SDS was performed according to Laemmli (1970).

Results

CK activity in spermatozoa and in seminal plasma

Fresh spermatozoa from chicken showed after 3–5 washes in isowash a specific CK activity of 1.5 ± 0.3 μmol CP min^{-1} mg^{-1} spermatozoan protein. No CK activity whatsoever was found in seminal plasma of chicken sperma. Human spermatozoa isolated and washed by centrifugation in Percoll (Arcidiacono *et al.*, 1983) showed a specific CK activity of 0.9 ± 0.4 μmol CP min^{-1} mg^{-1} of spermatozoan protein with considerable variation from sample to sample. In contrast to chicken, however, seminal plasma from man obtained from Percoll supernatants which were freed of residual spermatozoa and cellular debris did show CK activity (1.4 ± 0.4 μl CP min^{-1} ml^{-1} of seminal plasma).

Isoenzymes of CK in spermatozoa

Extracts of washed spermatozoa obtained by incubation with 0.2–0.5% Triton X-100 clearly showed after electrophoresis on cellulose polyacetate strips, the presence of a CK isoenzyme that comigrated with chicken brain type BB-CK (Fig. 1, lanes 3 and 2) as well as mitochondrial CK (MiMi-CK) known under these conditions to migrate towards the cathode. In addition, considerable myokinase (MK) activity was found in spermatozoa (Fig. 1, lane 3). The band corresponding to myokinase was demonstrated by omitting CP in the overlay reaction gel; the reaction was inhibited by the myokinase blocker diadenosine pentaphosphate (Ap5A) not shown here. The presence of MiMi-CK in chicken spermatozoa was visualized more clearly after quantitative extraction of MiMi-CK from sperm by a treatment known to extract mitochondrial CK from the inner mitochondrial membrane of heart mitochondria (Jacobus & Lehninger, 1973), that is, by swelling of the mitochondria in distilled water followed by extraction with phosphate buffer at pH 8.0 (Fig. 1, lane 4). The presence of both BB-CK and MiMi-CK, but not MM-CK, in spermatozoa from chicken is corroborated by immunoblots of total sperma, as well as of washed and partially extracted spermatozoa (Fig. 2). SDS extracts of both total sperma and washed spermatozoa from chicken show, after PAGE in SDS and staining for protein, a doublet of bands in the M_r range of 40 000 with apparent electrophoretic mobility similar to actin (Fig. 2a). After electrophoretic transfer of a parallel set of samples to nitrocellulose followed by incubation with specific anti-B-CK antibodies, the presence of brain-type B-CK subunits corresponding to the upper of the two protein bands (M_r = 43 000) was revealed in total sperma, washed

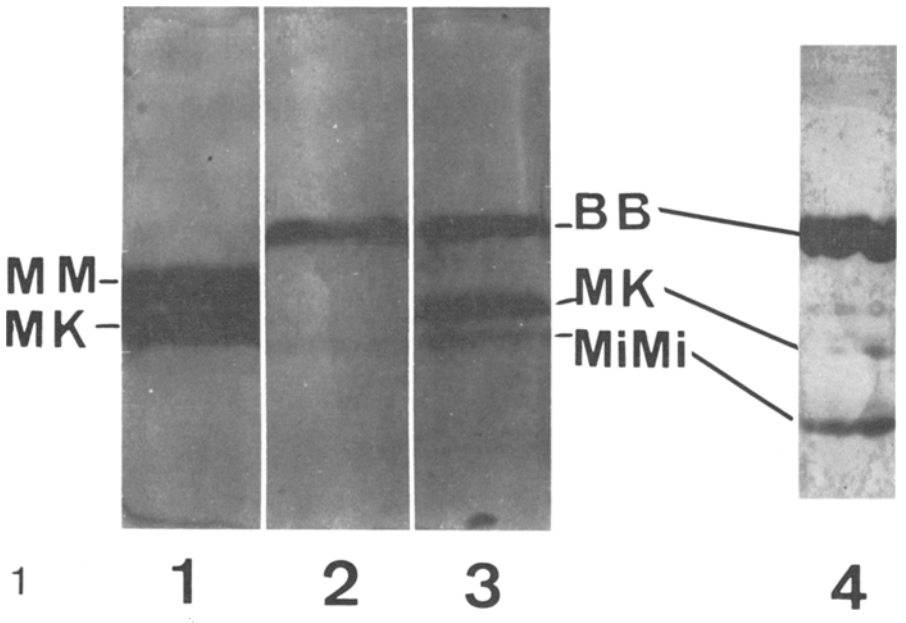

Fig. 1. Identification of creatine kinase isoenzymes and myokinase in spermatozoa. Extracts from (1) chicken skeletal muscle; (2) chicken brain; and (3) washed chicken spermatozoa, after electrophoresis on cellulose polyacetate strips and subsequent staining for CK activity. Lane 4 represents the CK isoenzymes released from washed chicken sperm by a treatment known to extract mitochondrial MiMi-CK from isolated heart mitochondria (see Experimental procedures). Myokinase (MK) activity remains after omission of CP, but is specifically inhibited by AP5A (see results). MM, muscle form of CK; BB, brain form of CK; MiMi, mitochondrial form of CK; MK, myokinase or adenylate kinase.

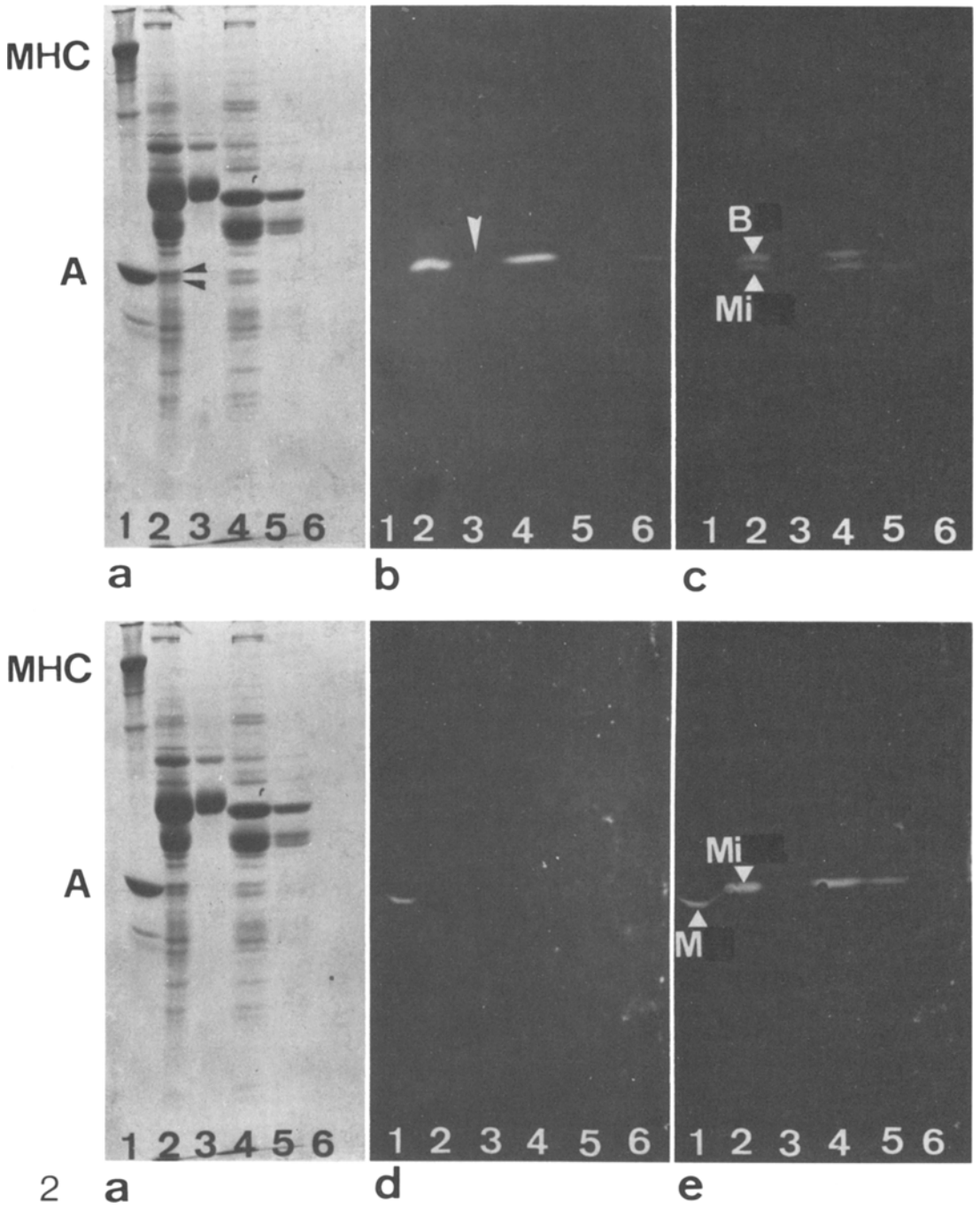

Fig. 2. Immunoblots showing subunit types of creatine kinase isoenzymes in spermatozoa from chicken after PAGE in the presence of SDS. (a) Protein bands of washed chicken myofibrils (1); total sperm (2); seminal plasma (3); washed sperm (4); pellet of sperm partially extracted by 0.1% Triton for 5 min (5); and supernatant thereof (6), all stained for protein with Coomassie Brilliant Blue. (b) Same samples as in (a) but after electrophoretic transfer to nitrocellulose and subsequent staining with anti-B-CK antibodies followed by FITC-conjugated second antibody. Note the presence of B-CK in total sperma (2), washed spermatozoa (4), and Triton extract (6). Note the absence of B-CK in chicken seminal plasma (3, white arrowhead). Black arrowheads (a2) pointing to the protein band doublet identified by immunoblotting as B-CK (upper band, apparent M_r = 43 000) and as Mi-CK subunits (lower band, apparent M_r = 42 000). (c) Same samples as in (b) but after staining for both B-CK and Mi-CK subunits of CK. Note the presence of both, B-CK and Mi-CK, in total sperma (2), and in washed spermatozoa (4). (d) Same samples as in (a) but after electrophoretic transfer to nitrocellulose and subsequent staining with anti-M-CK antibody. Note the absence of muscle type M-CK subunits in sperma and spermatozoa (2, 4). (e) Same samples as (d) after staining for both, M-CK and Mi-CK subunits of CK. Note again the presence of Mi-CK in total sperm (2), washed spermatozoa (4), in partially extracted spermatozoa (5). Note also the presence of M-CK (apparent M_r = 40 000) in washed chicken myofibrils (Wallimann *et al.*, 1984). MHC, myosin heavy chain; A, actin; M, muscle type subunit of CK; Mi, mitochondrial type subunit of CK.

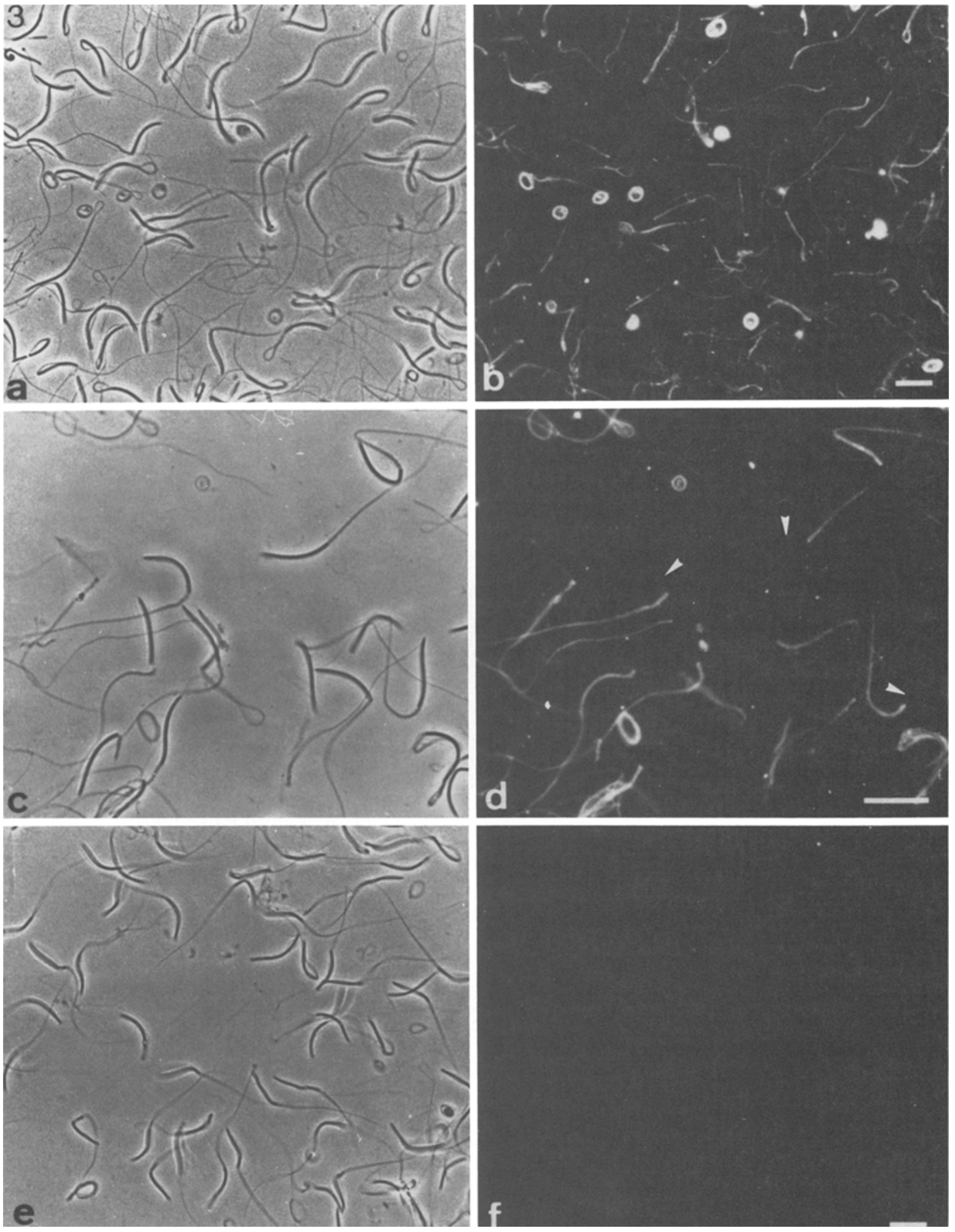

Fig. 3. Indirect immunofluorescence staining of chicken spermatozoa with anti-B-CK antibodies. Washed spermatozoa from roosters attached to glass slides permeabilized and fixed for 1 min with methanol at −20° C are shown after incubation with subunit specific anti-B-CK IgG (2 μg/μl) and subsequent staining with FITC-conjugated goat anti-rabbit IgG (a–d). Control using same concentration of control IgG followed by FITC-conjugated second antibody (e, f). Phase contrast (a, c, e) and fluorescence pictures (b, d, f). Arrowheads in (d) point to the location of the elongated chicken sperm heads which were not stained at all. Double-line staining is seen in the upper third of the spermatozoon tail just below the arrowhead on the left. (a, b, e, f) × 650; (c, d) × 1150. Scale bar, 10 μm.

spermatozoa and in the sperm extract but not in the seminal plasma while very little B-CK was left in Triton extracted spermatozoa (Fig. 2b). Anti-chicken B-CK antibodies previously absorbed with MM-CK did not show any cross-reactivity with M-CK of chicken myofibrils and reacted with a single band only of apparent M_r of 43 000 of total chicken sperma (Fig. 2b). Subsequent incubation of the anti-B-CK-stained blots with anti-MiMi-CK antibodies revealed a second single band (lower than B-CK) in total sperma, washed spermatozoa and partially extracted spermatozoa (Fig. 2c) which had an apparent M_r of 42 000 corresponding to the lower of the two protein bands (Fig. 2a). This demonstrates clearly the presence of both mitochondrial creatine kinase subunits as well as brain-type CK subunits (B-CK) in total sperma and washed spermatozoa from chicken. The absence of muscle type CK is demonstrated by immunoblots stained with specific anti-M-CK antibodies (Fig. 2d) where the only positive signal obtained was M-CK bound to the M-band of washed myofibrils showing an apparent M_r of 40 000 (Turner *et al.*, 1974; Walliman *et al.*, 1977, 1983a, b). As can be seen in Fig. 2, the mitochondrial Mi-CK subunit migrates with a lower apparent mobility than the M-CK subunit. Thus, the three CK subunits, B-CK, Mi-CK and M-CK can be identified, in addition to their specific reactivity with homologous anti-CK antibodies, by their different electrophoretic mobilities in SDS–polyacrylamide gels. In summary, B-CK as well as Mi-CK but no M-CK subunits were found in washed spermatozoa. No CK-subunits were found by these methods in seminal plasma from chicken. BB-CK, presumably present in spermatozoa as a generally soluble isoenzyme, is more readily extracted by Triton X-100 treatment than MiMi-CK (Fig. 2).

Creatine content of chicken sperma

The values for the total creatine content were determined as 3.8 ± 0.4 mM for chicken seminal plasma and 1.2–1.5 mM for extracts from washed chicken sperm resuspended to the original volume. Considering that a packed spermatozoan pellet from chicken sperma represents between 10–15% of the total sperma volume an intra-spermatozoan creatine concentration of approximately 8–15 mM may be estimated.

Localization of CK isoenzymes in spermatozoa

After incubating chicken spermatozoa with specific anti-B-CK antibodies (see immunoblots in Fig. 2), immunofluorescence-staining of the midpiece and tail region, but not of the heads, occurred in all spermatozoa (Fig. 3b). This can be demonstrated even better at higher magnification (Fig. 3d, white arrowheads point towards the elongated chicken spermatozoa heads) whereby the immunofluorescence staining could often be resolved into two discrete lines, especially within the upper third of the tails. Fluorescence also seems to get weaker towards the tail ends which are known to taper off slowly. This staining behaviour was independent of fixation (formaldehyde or methanol at −20° C). However, the intensity of staining was quite variable especially when Triton X-100 permeabilization was used. It became obvious that Triton-X-100, which had to be used to make the spermatozoa accessible to the antibodies, did remove, depending on the duration of the treatment, some of the BB-CK (see immunoblots in Fig. 2). Thus, the fluorescence signal obtained after Triton permeabilization is likely to represent staining of a more strongly bound fraction of B-CK presumably associated with the sperm tail ensheathment. Permeabilization of sperm membranes and concomitant fixation with −20° C methanol seemed to prevent leakage of soluble B-CK and was adopted as the method of choice in terms of fluorescence signal. Weak staining with anti-MiMi-CK was found in the midpiece neck region of the spermatozoa where the mitochondria are located (not shown here), however, this was also the location of some unspecific staining (stickiness) by control IgG (Fig. 3f). No staining whatsoever, even of damaged, swollen or partially disrupted sperm heads was found with antibodies by this technique indicating that BB-CK and MiMi-CK are both located exclusively within the entire length of the sperm tails and the mitochondria-rich midpiece region, respectively. Also, no specific staining was found without prior permeabilization either with Triton or cold methanol indicating that the CK isoenzymes are indeed located within the spermatozoa. Experiments are currently being carried out to determine at the electron microscope level the exact localization of BB-CK within spermatozoa.

Ultrastructural localization of mitochondrial CK

Immunogold staining of ultrathin frozen sections of gelatin-embedded suspension of chicken spermatozoa showed after labelling with anti-chicken mitochondrial CK antibodies heavy labelling of the mitochondria-rich middle piece of the spermatozoa (not shown). At higher magnification a clear antibody–gold labelling of the inside of the mitochondria was resolved (Fig. 4) whereas no staining of sperm tail regions lacking mitochondria was seen (Fig. 4, left hand). This result represents the first immunohistochemical localization of mitochondrial CK in mitochondria and indicates in accordance with biochemical data (Jacobus & Lehninger, 1973) that this CK isoenzyme is strictly restricted to mitochondria and that it seems to be associated with the inner mitochondrial membrane. However, even though the

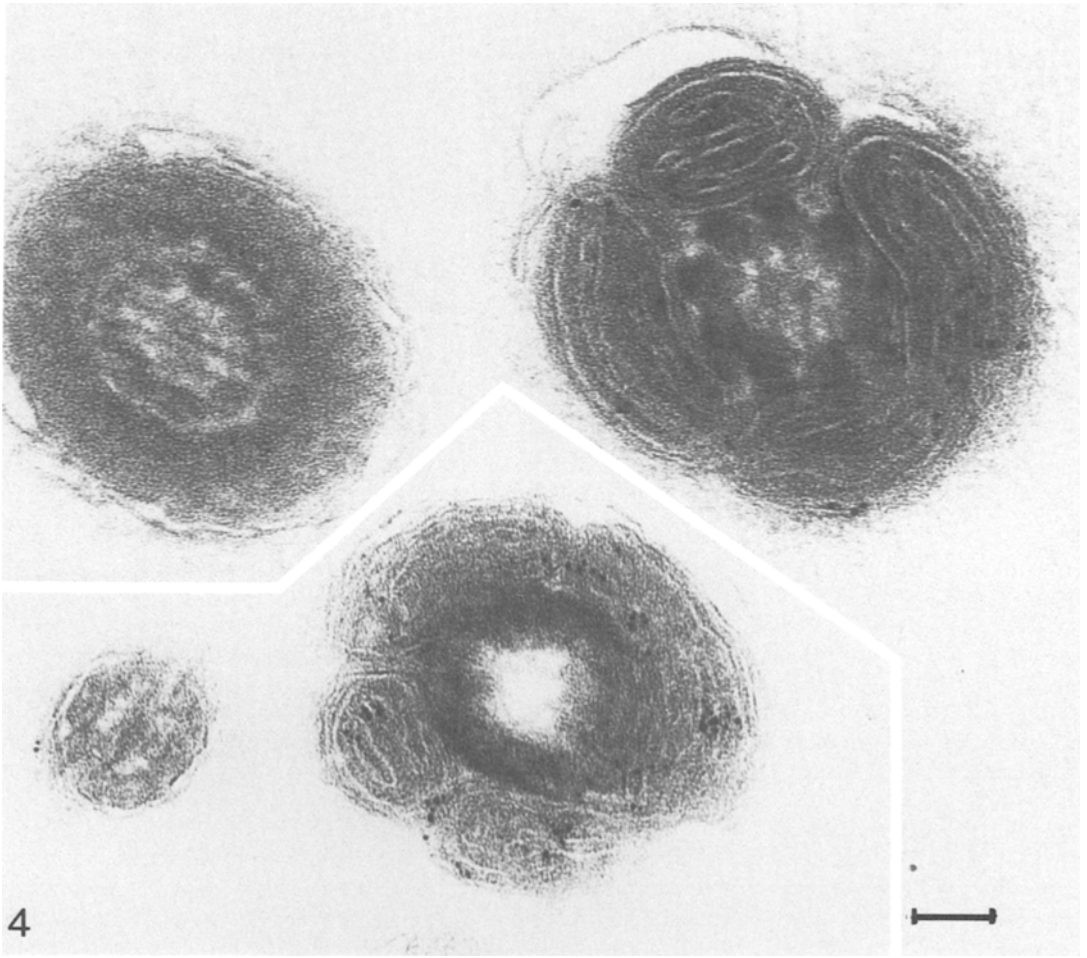

Fig. 4. Ultrastructural localization of mitochondrial CK in sperm. Ultrathin cryosection of a frozen fixed spermatozoa–gelatin suspension after staining with 1:100 diluted rabbit anti-chicken mitochondrial CK serum followed by goat anti-rabbit IgG–10 nm gold colloid complex. Note labelling of inside of mitochondria and absence of labelling in cross-sections with no mitochondria. × 100 000. Scale bar, 100 nm.

structural preservation of the sperm cross-sections after ultracryotomy is excellent one cannot resolve by this technique the questions of sidedness of mitochondrial CK localization at the inner mitochondrial membrane. Experiments to demonstrate the sidedness of mitochondrial CK localization as well as to localize BB-CK on an electron microscopic level within the spermatozoan tail are now in progress.

Human spermatozoa

Similar results were obtained with human spermatozoa with the one difference that seminal plasma from man contained significant BB-CK activity (1.4 ± 0.4 μmol CP min^{-1} ml^{-1} seminal plasma) which is shown here by immunoblotting and staining with anti-human B-CK antibodies (Fig. 5). In Percoll-washed human spermatozoa, like in spermatozoa from chicken, the presence of both BB-CK and MiMi-CK was demonstrated by the immunoblot method (Fig. 5).

Creatine kinase and sperm motility

After chicken spermatozoan motility had been blocked by 50 μm carboxyatractyloside and 20 mM deoxyglucose, being inhibitors of both the ATP–ADP translocator and glycolysis respectively, some motility was regained by the addition of 10 mM CP plus 1 mM ADP in the presence of 0.5 mM Ap5A, although not to the same degree as with direct addition of ATP. In both cases, however, the regained motility was always lower than in untreated controls.

Sperm motility was severely affected by the addition of 15 μM dinitrofluorobenzene (DNFB) to a ten times diluted (BSA–PBS) spermatozoa suspension as shown very recently by Tombes & Shapiro (1985).

Discussion

The presence of both myokinase and creatine kinase in spermatozoa, both of which represent powerful ATP regenerating systems in muscle, imply strongly

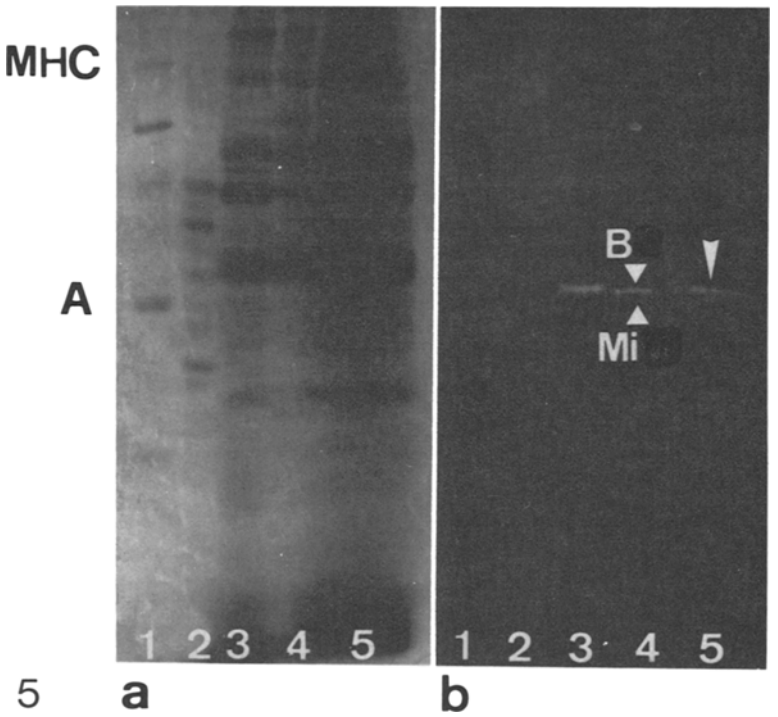

Fig. 5. Identification by immunoblots of subunits types of CK isoenzymes in human spermatozoa and seminal plasma. Washed skeletal muscle myofibrils (1); diluted brain extract (2); total sperma (3); Percoll-gradient washed spermatozoa (4); and seminal plasma (5) all from humans, after electrophoresis in 10% polyacrylamide gels in the presence of SDS stained for protein (a). Parallel gel with the samples as in (a) after electrophoretic transfer to nitrocellulose and incubation first with anti-human B-CK followed by anti-human Mi-CK antibodies, subsequently stained with FITC-conjugated second antibody (b). Note the presence of B-CK (B) and a faint lower band corresponding to Mi-CK (Mi) in isolated spermatozoa (b, lane 3). Note also the presence of considerable amounts of B-CK in seminal plasma (b, lane 4). MHC, myosin heavy chain; A, actin.

that these systems may also be of physiological importance for sperm motility since these cells also have an extremely high energy expenditure. CP has long been regarded as a buffer of ATP levels in muscle cells and the fact that in spermatozoa both the brain isoform (BB-CK) and the mitochondrial isoform (MiMi-CK) of CK are present simultaneously is interesting since, so far, mitochondrial CK has only been reported in skeletal muscle, heart muscle and in brain, which are all organs with high energy expenditure (for review see Jacobus & Ingwall, 1980). Under certain conditions, where ATP has a long diffusion path and the ATPase rate is high (Meyer *et al.*, 1984), a 'phospho–creatine shuttle' mechanism may enhance efficiency (Wallimann, 1975; Saks *et al.*, 1978; Bessman & Geiger, 1981; Wallimann & Eppenberger, 1984; Wallimann *et al.*, 1984). That this may be operating here is suggested by the presence in spermatozoa both of creatine and of two spatially segregated CK isoenzymes, MiMi-CK being located within the mitochondria in the midpiece region and BB-CK being distributed over the entire length of the sperm tail. According to this scheme ATP is transphosphorylated by MiMi-CK in the mitochondria in the sperm midpiece, yielding CP which is then transported to sites of ATP utilization (e.g. dynein–tubulin system). Subsequently, BB-CK (located throughout the sperm tail), generates the required ATP from this CP and ADP.

In contrast to seminal plasma from chicken which does not contain any CK, seminal plasma from man was found to contain significant amounts of BB-CK, the origin of which has been suggested to be multiglandular, namely from the prostatic gland (Kavanagh & Darby, 1983) and the seminal vesicle (Soufir, 1979). It should be pointed out here that hens after mating can store spermatozoa over long periods of time and release them when needed for fertilization of the eggs; the lack of an additional external CK supply in seminal fluid of roosters may be related to this physiological speciality. CK in human seminal plasma may be important as an additional, external back-up system supporting the immediate energy demands of spermatozoa which have to reach their target for viability reasons within 24 h of ejaculation.

The importance of CK and CP in sperm motility is supported by preliminary data which indicate that sperm which had been blocked by carboxyatractyloside, a specific blocker of the mitochondrial ATP–ADP translocase system resume some motility upon addition of 1 mM ADP and 10 mM CP. Low concentrations of DNFB (10–20 μM) an agent known at these

concentrations to block creatine kinase activity rather specifically without interfering with ATPase (Wallimann *et al.*, 1984) had a strong inhibitory effect on sperm motility. These results suggest a linkage between sperm respiration and motility mediated by mitochondrial CK in the midpiece region and BB-CK in the tail region through a CP-shuttle. In addition an inhibition of human spermatozoa by anti-B-CK antibody, but not by anti-M-CK antibody, has been reported (Chapman *et al.*, 1980). Since at this time no information was available on the existence of BB-CK within spermatozoa the antibody was interpreted by these authors (Chapman *et al.*, 1980) to affect the BB-CK present in human seminal plasma only. The same investigators suggested an inhibition of seminal plasma BB-CK by antibodies found in considerable concentrations in the blood of some infertile women.

It is interesting to mention that a positive correlation between the number of spermatozoa and CK activity in human seminal plasma (Menaché *et al.*, 1976) and a statistically significant difference of CK activity in seminal plasma between men with normal spermiograms and oligoasthenospermia, yet with wide overlapping of the individual values, has been reported (Asseo *et al.*, 1981). The determination, however, of CK activity in human seminal plasma seems to be of limited diagnostic value for male infertility (Gonzales-Buitrago *et al.*, 1980), while measurement of BB-CK activity in seminal plasma, prostatic fluid or urine has been suggested to be a valuable diagnostic tool for prostatic carcinoma (Chapman *et al.*, 1980; Henderson & Nealon, 1981; Edwards *et al.*, 1982). Based on our results we believe that the determination of CK activity of isolated spermatozoa as opposed to seminal plasma may be of more clinical potential in the diagnosis of male infertility. Creatine kinase isoenzymes within spermatozoa may also prove a system to be considered seriously as a target for manipulation of sperm viability either in the direction of interfering with infertility or of developing new means of birth control.

We postulate that the CK–CP system in spermatozoa is not merely a simple back-up system, but is directly involved in sperm motility by forming functionally coupled microcompartments with ATP-generating sites on the one hand and ATP-utilizing sites on the other hand, where communication between these sites is mediated by CP (for review see Wallimann & Eppenberger, 1985).

Note added in proof. Recently Tombes *et al.*, (1984) showed, in agreement with our results, that CK activity is essential for optimal respiration and motility of sperm and have demonstrated a CP-shuttle operating in sea urchin sperm (Tombes & Shapiro, 1985).

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