FUNCTIONS OF CREATINE KINASE ISOENZYMES IN SPERMATOZOA

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ABSTRACT

Spermatozoa have a defined function, that is, the fertilization of an egg. To achieve this goal, spermatozoa have to move actively toward the egg and inject the male pronucleus into the egg. Therefore, sperm motility is one of the crucial determinants for male fertility. Surprisingly, however, structure and energy metabolism can differ substantially between spermatozoa from different species. Here, we report on the presence of high-energy guanidino compounds and the presence of guanidino kinases in spermatozoa of different animal species, focusing mainly on creatine kinase (CK) isoenzymes. Spermatozoa from lugworm, sea urchin, rooster and a number of mammals, including man, contain a cytosolic CK isoenzyme, in most cases brain-type B-CK, localized to the sperm tail. In addition, at least one mitochondrial CK isoenzyme was identified and specifically localized in the mitochondria containing midpiece. Specific inhibition of CK was shown to abolish flagellar movement in the distal third of the sperm tail from sea urchin. These experiments demonstrate in an *in vivo* cell model system the physiological importance of the CK isoenzymes participating in a phospho–creatine shuttle to overcome the diffusional limitations of adenosine nucleotides along the sperm tail. By comparison, the relative importance of CK for sperm function may range from absolutely essential, in sea urchin and rooster, to marginal, in some mammals, some of which do not contain CK in their sperms at all, for example, bull and boar. In the latter case, however, systems like fermentation and/or the adenylate kinase system compensate for the lack of CK and/or take over the function of CK.

I. GENERAL INTRODUCTION

Although spermatozoa are structurally streamlined and functionally minimized cells with a sole defined function, that is, the fertilization of an egg, the structure and energy metabolism of sperm cells from different species show a remarkable variability (Baccetti and Afzelius, 1976). These features are all optimized for the actual fertilization process and the environment where fertilization takes place (see

Chapter VI). For example, due to their entirely endogenous supply of energy by phospholipids (Mite and Yasumasu, 1983), the "primitive" sperm cells of sea urchins are well suited for the fertilization of simultaneously spawned eggs in the fully oxygenated ocean. However, they differ vastly in structural and metabolic terms from mammalian spermatozoa which swim up through the female tract in a "sheltered" environment, albeit under low oxygen tension or even anoxia, where they may survive for hours or even weeks, thanks to a supply of external energy sources (see Chapter V).

Therefore, it is not surprising that sperm cells from different species also differ in the content of guanidino kinases, a family of enzymes involved in the regeneration of ATP (Watts, 1971). This family comprises creatine kinase (CK), arginine kinase (ArgK), guanidinoacetate kinase, lombricine kinase, taurocyamine kinase, hypotaurocyamine kinase, and opheline kinase; but most sperm cells with the exception of mollusks and arthropods spermatozoa use CK only (see Chapter V; Watts, 1971; Ellington, 1989). These enzymes catalyze the reversible transfer of the N-phosphoryl group from the respective phosphorylguanidines to ADP in order to regenerate ATP. In this review, we will focus mostly on CK. In general, CK isoenzymes are found in cells with high and fluctuating energy demand, like skeletal and cardiac muscle, brain, photoreceptor cells, electrocytes, and spermatozoa (for review see Wallimann et al., 1992). So far five different CK isoenzymes have been identified in vertebrates; two are found in the cytosol and form exclusively dimeric molecules (MM-CK, MB-CK, and BB-CK; M for the "muscle" isoform; B for the "brain" isoform). The other two CK isoenzymes are located in the mitochondrial intermembrane space and form homo-octameric as well as homo-dimeric molecules (Mi_a-CK or ubiquitous and Mi_b-CK or sarcomeric isoenzyme; for a review see Wyss et al., 1992). In most tissues, cytosolic as well as mitochondrial CK isoenzymes are co-expressed. Both, phospho–creatine (PCr) and CK isoenzymes have been identified in spermatozoa of different species (see below; for review see Tombes and Shapiro, 1989).

Over the past years, a concept for multiple functions of the CK/PCr-system have been proposed, such as (1) an ATP buffering function to maintain a high ATP/ADP ratio in the cell, thus increasing the thermodynamic efficiency of energy production and energy utilization (Meyer et al., 1984), (2) a regulatory function for respiration and glycogenolysis by triggering the inorganic phosphate level (Meyer et al., 1984; Kamp, 1992; Wallimann et al., 1992), and (3) an energy transport function that would facilitate energy transfer within a cell (Saks et al., 1978; Bessman and Geiger, 1981; Bessman and Carpenter, 1985; Wallimann and Eppenberger, 1985; Wallimann et al., 1992).

While the temporal energy buffering and metabolic regulatory roles of CK are widely accepted, the spatial buffering or energy transport function, that is, the shuttling of PCr and creatine (Cr) between sites of energy utilization and energy demand, is still being debated and has elicited some controversy, especially as far as *in vivo* saturation-transfer ^{31}P-NMR measurements are concerned (for a recent

overview concerning the possible problems associated with *in vivo* ^{31}P-NMR see Wallimann, 1996).

There is, however, much circumstantial evidence supporting the latter role of CK, including the distinct, isoenzyme-specific subcellular localization of CK isoenzymes, the isolation and characterization of functionally coupled *in vitro* microcompartments of CK with a variety of cellular ATPases, and the observed functional coupling of mitochondrial oxidative phosphorylation with mitochondrial CK (Jacobus, 1985; Wallimann et al., 1992; for an overview over the field see the articles in the special volume 133/134 of Mol. Cell. Biochem., 1994). New insight concerning the functions of the CK/PCr-system has additionally been gained from transgenic mice lacking M-CK (van Deursen et al., 1993) and by the investigation of CK localization and function in certain highly specialized non-muscle tissues and cells, such as electrocytes, sperm, retina photoreceptors, brain and neuronal cells, kidney cells, salt glands, myometrium, placenta, pancreas, thymus, thyroid, intestinal brush-border epithelial cells, endothelial cells, cartilage and bone cells, macrophages, blood platelets, tumor and cancer cells (for review see Wallimann and Hemmer, 1994). In addition, the crystal structure of the first guanidino kinase, the one of octameric Mi_b-CK from chicken heart, has been solved at atomic resolution (Fritz-Wolf et al., 1996). The structure is compatible with the energy channeling function proposed for this enzyme (Wallimann et al., 1992).

A major disagreement over the PCr-shuttle hypothesis also concerns the question of whether the diffusion of ATP, or more importantly of ADP, from cellular sites of energy production to sites of energy consumption or vice versa, respectively, is limited in cells-with high and fluctuating energy demand, or not. ADP is present in resting cells at concentrations of 15–30 μM only and does usually not increase by more than 10-fold upon cell activation. This also holds true for spermatozoa (see Chapter II; Christen et al., 1983a, 1983b) where the calculated free ADP concentration rises from 9 μM to only 114 μM upon sperm activation (van Dorsten, 1996; van Dorsten et al., 1997). In addition, spermatozoa are highly polarized cells with long tails, usually ranging from 40–200 μm in length, and with ATP sinks (axonemal dynein ATPase) along the entire length of the sperm flagella. Therefore, diffusion limitations of both ATP, and even more so of ADP, are to be expected. This is especially true for spermatozoa that depend entirely on energy production by the mitochondria. In particular, sea urchin spermatozoa represent an ideal model system suited for testing the PCr-shuttle hypothesis *in vivo*, since their motility depends upon oxidative metabolism in the mitochondrion at the base of the sperm head. Additionally, sea urchin sperm can be collected in large quantities and are amenable to biochemical analysis because they can be activated synchronously and manipulated in physiological terms by simply diluting spawned sea urchin sperm into sea water and by changing ionic and pH conditions of the sea water, respectively (Christen et al., 1982; Trimmer and Vacquier, 1986; Shapiro, 1987).

II. SEA URCHIN SPERM ACTIVATION AND METABOLISM: A UNIQUE *IN VIVO* MODEL SYSTEM TO TEST THE PCr-SHUTTLE HYPOTHESIS

Influx of sodium from the sea water into the sperm cell via an Na^+/H^+ exchanger (Nishioka and Cross, 1978) leads to elevation of the intracellular sperm cell pH from 7.0 to its resting value of 7.5 (Christen et al., 1982). In turn, this leads directly or indirectly to an activation of the pH-sensitive dynein ATPase (Christen et al., 1983a) which drives flagellar movement (Gibbons and Gibbons, 1972). As a result of activation of sperm motility, the consumption of high-energy phosphate increases (Christen et al., 1983b) and mitochondrial respiration is then turned on (Christen et al., 1982). Most importantly, the rates of high-energy phosphate metabolism of sea urchin sperm, thriving exclusively on mitochondrial oxidative phosphorylation of fatty acids, derived either from phosphatidylcholine (Echinoida) or triglycerides (Arbacioida) (see Mita et al., 1994), is highly regulated in response to the energy demand in the flagellum. The dynein ATPase consumes virtually all cellular ATP for cell motility (Christen et al., 1982, 1983a). Motile sea urchin sperm respire maximally, that is, O_2 consumption by the single mitochondrion at the base of the sperm head does not increase with addition of uncoupling agents (Christen et al., 1983a). Thus, since in sea urchin sperm all of the high-energy phosphate is supplied entirely by the mitochondrion and more than 95% of the ATP is used by the dynein ATPase in the 40–50 μm long flagellum, the system must be capable of providing sufficient ATP to the distal end of the flagellum, which is rather far away from the mitochondrion. Based on the high rates of ATP utilization by sea urchin sperm flagella (Christen et al., 1982, 1983a) and the fact that diffusion may be severely restricted due to the highly occupied space within the axoneme, it was suggested that ATP- and especially ADP-diffusion alone may not be sufficient for adequately supplying energy to the flagellum of the long sperm tail (Tombes et al., 1987). A similar situation can be nicely visualized in spermatozoa from the marine lugworm *Arenicola marina*, where diffusion rates and distances have been calculated as depicted in Figure 1.

Therefore, the presence of distinctly localized CK isoenzymes in sperm (see below) may be a clue for the role of the PCr/CK system to facilitate high-energy phosphate transport in these highly polar cells.

A. Creatine Kinase Isoenzymes in Sea Urchin Sperm

Arginine kinase (ArgK) was considered as the invertebrate guanidino kinase, while CK isoenzymes were supposed to be entirely restricted to vertebrates (Richterich et al., 1968). However, echinoderms turned out to contain CK, too. For example, sea urchins, expressing a unique CK in sperm (see below), ArgK in eggs and both guanidino kinases in somatic cells, seem to represent an evolutionary *transition* point for guanidino kinases (see Chapter VI; Tombes and Shapiro, 1989).

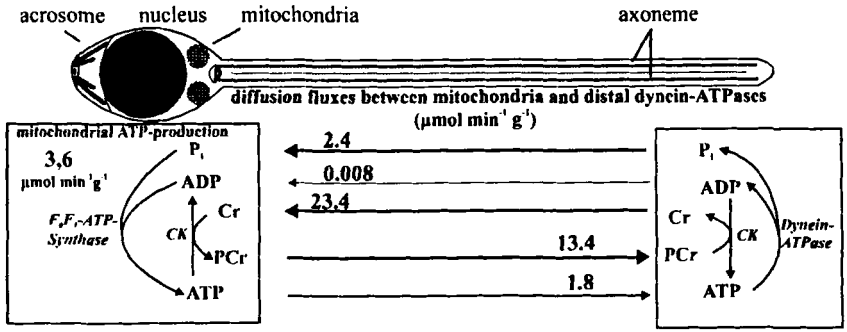

Figure 1. Diffusion flux rates of substrates in a primitive sperm from the marine lugworm *Arenicola marina*. Comparison of mitochondrial ATP production with estimated radial diffusion flux between mitochondria and distal dynein ATPases in motile sperm of the lugworm *Arenicola marina*. Mitochondrial ATP production was calculated from oxygen consumption and the intracellular diffusion (Q) of metabolites by the standard equation described by Jacobus (1985). Details of calculation are described by Kamp et al. (1995). The estimated flux of free ADP is about 500 times below the rate of mitochondrial ATP production, whereas the Cr flux would allow an unlimited transfer of high-energy phosphate from the mitochondria to the distal dynein ATPases.

Sperm from several species of sea urchins (order of Arbacioida as well as Echinoida) show high concentrations of PCr (Yanagisawa, 1967) and very high activities of CK, similar to that of skeletal muscle, as their sole guanidino kinase system (Fujimaki and Yanagisawa, 1978; Harumi et al., 1992). For comparison of ArgK and CK activities of different animal species see Table 1 of Tombes and Shapiro (1989) as well as Table 1.

Two distinctly localized CK isoforms at approximately equal activities are found in sea urchin sperm. A 145 kDa flagellar tail CK (TCK) is located in the long sperm tail (Wothe et al., 1990), and another CK isoform with a M_r of approximately 47 kDa, showing marked microheterogeneity, is located exclusively in the sperm head fraction (Tombes and Shapiro, 1985). The latter CK isoenzyme, representing mitochondrial CK [Mi-CK] (Wyss et al., 1995), is situated specifically within the single mitochondrion at the base of the sea urchin sperm head (Tombes and Shapiro, 1987). Sea urchin sperm Mi-CK, as vertebrate Mi-CK (Schlegel et al., 1988a) also forms octamers (Wyss et al., 1995) with a central hollow channel (for review see Wyss et al., 1992).

Flagellar TCK was shown to result from a gene triplication. Each of the three contiguous CK segments of TCK is homologous to vertebrate CKs. TCK is both a cytosolic enzyme and a principal component of sperm tail membrane preparations (Quest and Shapiro, 1991). Recently, it could be shown that myristoylated and non-myristoylated forms of TCK exist side by side in the sea urchin sperm

Table 1. Enzyme Activities in Spermatozoa

		CK		*GAPDH*		*LDH*		*CS*		*HOADH*	
mammals	rat	1.2 ± 1.2	(6)	45 ± 7	(6)	54 ± 11	(5)	6 ± 4	(4)	1 ± 0.4	(3)
	stallion	1.9 ± 1.3	(8)	55 ± 21	(9)	13 ± 3	(8)	1 ± 0.5	(8)	0.5 ± 0.1	(4)
	bull	<0.1	(3)	48 ± 37	(10)	43 ± 17	(9)	4.1 ± 2.4	(9)	0.5 ± 0.1	(9)
	boar	<0.1	(3)	9 ± 8	(16)	4 ± 2	(8)	0.2 ± 0.1	(8)	0.3 ± 0.2	(9)
bird	turkey	700 ± 143	(3)	16 ± 3	(3)	15 ± 4	(3)	19 ± 4	(3)	6 ± 2	(3)
fish	carp	40 ± 12	(5)	8 ± 1	(5)	16 ± 4	(5)	3 ± 1	(5)	2 ± 0.4	(5)
echinoderm	sea urchin	382 ± 123	(3)	1 ± 1	(3)	<0.1	(3)	33 ± 9	(3)	36 ± 16	(3)
annelid	lugworm	64 ± 9	(3)	28 ± 4	(3)	[a]8 ± 2	(3)	1.8 ± 0.4	(3)	15 ± 4	(3)

Source: Kamp et al. (1996).

Notes: Enzyme activities were measured in cell extracts after sonication and centrifugation, in the solution of resuspended cell fragments using standard enzymatic assays (Bergmeyer, 1983; Kamp et al., 1995). The activities are expressed in μmol min^{-1} g^{-1} cell wet weight (25°C) and are means ± S.E.M. with the number of independent observations in parentheses. CK = creatine kinase (EC 2.7.3.2); GAPDH = glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); LDH = lactate dehydrogenase (EC 1.1.1.28); CS = citrate synthase (EC 4.1.3.7); HOADH = hydroxyacyl-CoA dehydrogenase (EC = 1.1.1.35); [a] = alanopine dehydrogenase.

flagellum and that myristoylation is essential for efficient association with liposomes (Quest et al., 1992, 1997). In addition, TCK was also suggested to be a microtubule-associated protein (Tombes et al., 1988). Thus, like in muscle and other cells, CK isoenzymes are compartmentalized in an isoenzyme-specific manner also in sea urchin sperm cells. Part of the cytosolic flagellar TCK is located at subcellular sites of energy consumption (axonemal dynein ATPase) or is bound to the plasma membrane, whereas the mitochondrial Mi-CK isoform is strictly confined to the mitochondrion. Therefore, if one assumes that ATP generated in the mitochondrion by oxidative phosphorylation is immediately transphosphorylated to PCr, these data are suggestive for a CK shuttle from the mitochondrion to the flagellum (Wyss et al., 1995).

B. Phospho–creatine Shuttle between Mitochondrion and Tail: Effects of CK Inhibition on Sea Urchin Sperm Motility and Respiration

Experiments performed with the membrane-permeant reagent, DNFB (2,4-dinitro-1-fluorobenzene), a CK inhibitor forming a covalent derivative with the single "essential" cysteine residue (Mahowald et al., 1962; Furter et al., 1993), showed an unusual specificity for CK in intact sea urchin sperm, which was confirmed by ^{3}H-DNFB labeling patterns of sperm proteins on SDS polyacrylamide gels (Tombes and Shapiro, 1985). While 10 μM DNFB inhibited 90–95% of sperm CK activity, a concentration of 20–30 μM of the same reagent was needed to inactivate sperm dynein ATPase, myokinase, or coupled sperm respiration, whereas FCCP-uncoupled respiration was resistant to even higher concentrations of the inhibitor (Tombes and Shapiro, 1985). However, the most striking effect was that in parallel with the inhibition of sperm CK by DNFB, a clear and progressive inhibition of sperm motility patterns was also observed such that flagellar bending got restricted to the proximal parts of the tail whereas the distal part became stiff (Tombes and Shapiro, 1985).

If the high concentrations of CK and PCr in sperm would merely equilibrate energy stores, rather than to serve as an obligatory intermediate between mitochondrion and the tail to maintain steady state motility and respiration, these authors would not have obtained qualitative changes in motility patterns, since ATP could still have diffused along the tail. A subsequent detailed study of the flagellar wave attenuation observed with DNFB and a theoretical analysis of high-energy phosphate diffusion revealed that sperm motility is impaired (Tombes et al., 1987), so that the distal third to half of the flagellum becomes progressively stiffer as CK gets inhibited (Figure 2).

Most importantly, the functional deficit is overcome when DNFB-treated spermatozoa are demembranated by detergent and reactivated to swim with exogenously added ATP (Tombes et al., 1987), indicating that the flagellar motors were still intact after treatment with the CK inhibitor. In addition, theoretical analysis of high energy phosphate diffusion from the sperm mitochondrion to the distal tail

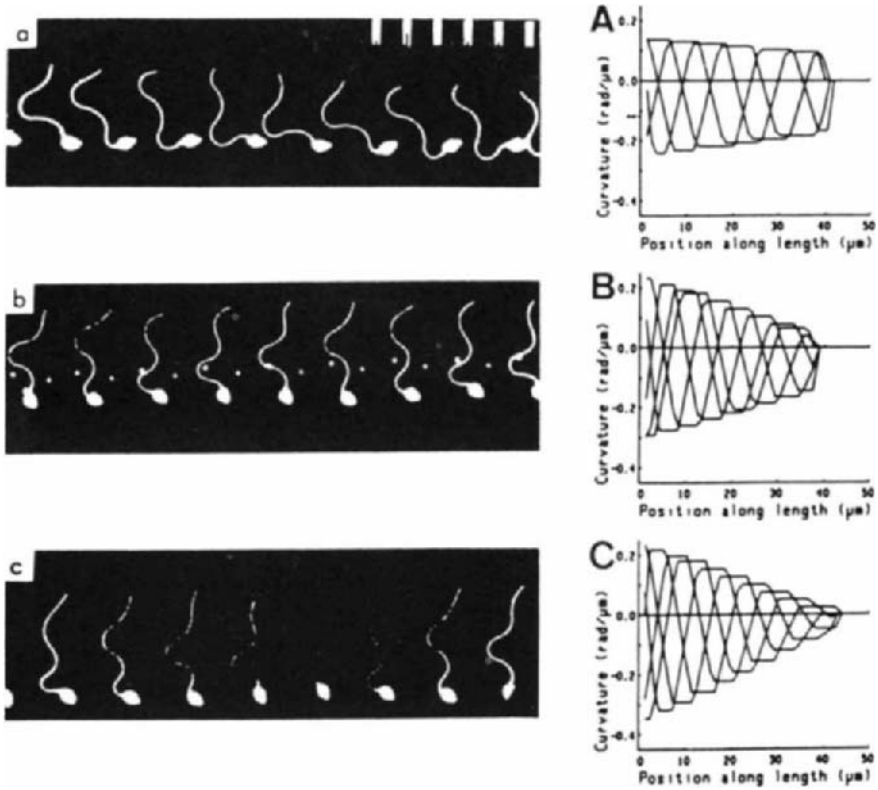

Figure 2. Effect of creatine kinase inhibition on flagellar wave bending of sea urchin spermatozoa. *Left panel*: examples of photographs of moving sea urchin spermatozoa taken on film with strobe flashes at 120 Hz. The scale marker (in a) represents 10 μm intervals. (a) live control spermatozoa; (b) and (c) same but treated with 15 μM DNFB to specifically inhibit CK. *Right panel*: analysis of flagellar bending waves after digitization, filtering and differentiation to obtain plots of curvature versus position along the length of the flagellum. The sperm head would be at a position to the left from 0 μm and the distal end of the sperm tail to the right at 40–50 μm. (A) Wave bending analysis of control sperm shown in (a); (B) and (C) wave bending analysis of sperm (a) and (c), respectively, after treatment with 15 μM DNFB for different lengths of time. Note that the distal end of the sperm tails, at right hand side of (B) and (C), are affected significantly, compared to the control (A), indicating diffusional limitations of ATP to the distal tail end, which stops bending, and of ADP from the tail back to the mitochondrion, as a consequence of CK inhibition (reprinted from Tombes et al., 1987b, with copyright permission from the Biophysical Journal).

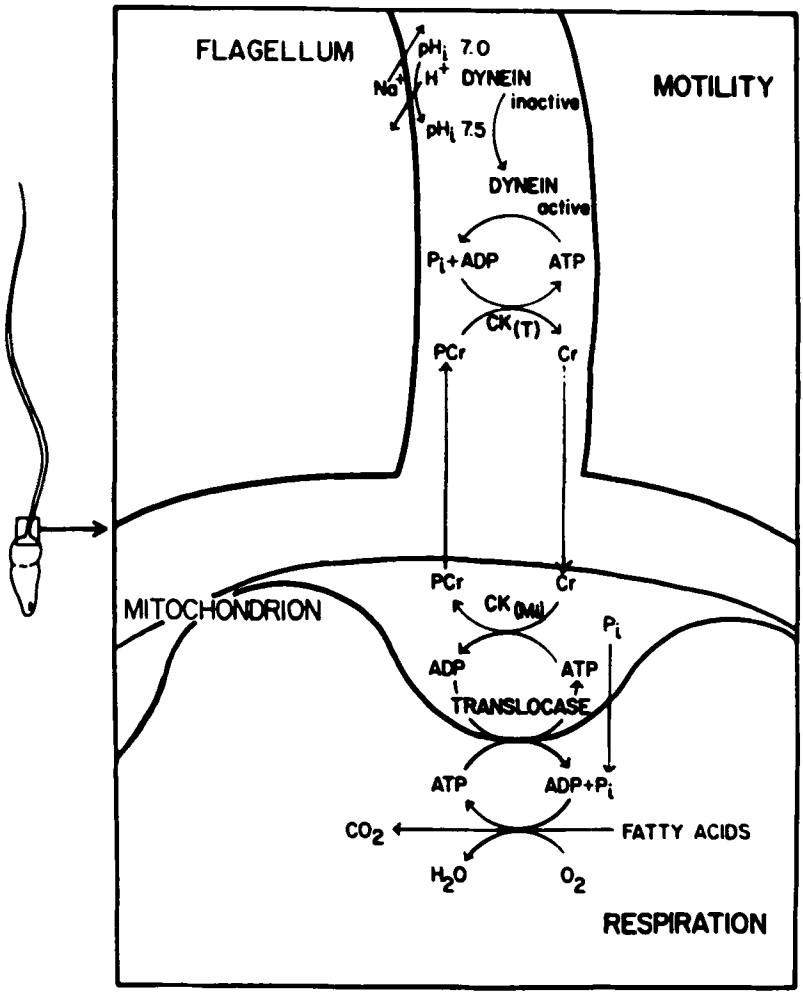

Figure 3. The phospho–creatine shuttle in sea urchin sperm. Model for the activation of sea urchin sperm motility and high-energy phosphate mediated energy flux, facilitated by mitochondrial $CK_{(Mi)}$ and flagellar tail $CK_{(T)}$ (read sequence of events for sperm activation from top to bottom of the enlarged drawing of the flagellar-mitochondrial junction of a whole sea urchin sperm, shown to the left). After spawning of sperm into sea water, sea urchin spermatozoa are activated by a number of sequential events involving an influx of sodium through the Na^+/H^+ exchanger, and a concomitant increase in pH from resting pH 7.0 to activating pH 7.5, resulting in the activation of the dynein ATPase and thus of cell motility. The consumption of high-energy phosphates by the dynein ATPase in the tail activates mitochondrial respiration, with PCr and Cr, as well as flagellar tail $CK_{(T)}$ and mitochondrial $CK_{(Mi)}$, mediating the energy flux as a PCr/Cr shuttle between the mitochondrion and the flagellar dynein ATPase (for details see text). The model suggests that PCr, Cr and inorganic phosphate (P_i) diffuse between the mitochondrion and the tail, overcoming the diffusional limitations of adenine nucleotides along the sperm tail (see also Figure 1). Reprinted from Tombes and Shapiro, 1985, with copyright permission from Cell.

(Tombes et al., 1987; see Figure 1) also strongly supports an involvement of the PCr-shuttle in sea urchin spermatozoa that is schematically visualized in Figure 3 (Tombes and Shapiro, 1985).

The importance of the PCr-shuttle in sea urchin sperm has recently been corroborated by *in vivo* ^{31}P-NMR saturation transfer measurements comparing the CK-mediated reaction flux of inactive versus sea water activated sperm (van Dorsten, 1997). In these experiments, where sperm motility and respiration were tested in parallel with the CK-mediated reaction flux, a significant increase of CK reaction flux by a factor of more than 10-fold, as well as a significant increase of estimated free ADP, from 9 μM to 114 μM, was observed upon sperm activation (van Dorsten et al., 1997).

In conclusion, the above data provide convincing evidence for a PCr-shuttle in sea urchin sperm to facilitate the directional flow of energy-rich phosphate from the mitochondrion to the axoneme and of Cr from the axoneme back to the mitochondrion (see schematic sperm PCr-shuttle model depicted in Figure 3; Tombes and Shapiro, 1985). The isoenzyme-specific localization of the sperm CK in different cellular compartments, that is, at sites of energy production as well as energy utilization, and the functional coupling of Mi-CK with mitochondrial respiration and of TCK with the dynein ATPase, allows for subcellularly compartmentalized metabolic events that are connected by the faster diffusing PCr and Cr, compared to ATP and ADP, respectively (see Figure 1, where an example of diffusional rates of relevant substrates is visualized schematically in a sperm cell).

Thus, sea urchin sperm turned out to be the best *in vivo* example and model system for the energy transport function of the PCr-shuttle (Bessman and Carpenter, 1985; Wallimann and Eppenberger, 1985; Wallimann et al., 1992).

C. Comparison of Mitochondrial Ultrastructure of Inactive versus Activated Sea Urchin Sperm

If, as suggested above, the PCr-shuttle plays an important role for energy transfer in sea urchin sperm, one may expect mitochondrial Mi-CK to be tightly coupled to oxidative phosphorylation for immediate transphosphorylation of mitochondrial matrix-generated ATP into PCr which then diffuses as the energy carrier into the sperm tail (see Figures 1 and 3). Mi-CK has been localized in muscle, brain, and retina at mitochondrial contact sites, where inner (IM) and outer mitochondrial membranes (OM) are in close apposition, as well as along the cristae membranes, both by biochemical fractionation methods (Kottke et al., 1991) and immuno electron microscopical localization (Schlegel et al., 1988b; Wegmann et al., 1991). Furthermore, an increase in the frequency of such contact sites, supposedly consisting in part of porin of the OM, ATP/ADP-translocator of the IM and octameric Mi-CK in between, all together could form a multienzyme complex for vectorial channeling of high-energy phosphates (Wallimann et al., 1992). This has been observed to occur upon activation of isolated mitochondria or cultured cells (for

review see Brdiczka and Wallimann, 1994). Therefore, since in sea urchin sperm respiration is enhanced by up to 50 times within one second after spawning into sea water (Christen et al., 1983a), a dramatic increase in the frequency of these contact sites was anticipated in these cells.

A first attempt to show this by cryofixation and freeze-fracturing, as elegantly demonstrated earlier with isolated mitochondria (Knoll and Brdiczka, 1983) or intact cells (Brdiczka et al., 1986), was not successful with intact sea urchin spermatozoa. Almost no deflections of the fracture plane between IM and OM were encountered because most of the fractures were either equatorial through the mitochondrion or entirely in the plasma membrane (Piendl, 1996).

In an alternative attempt to visualize mitochondrial IM/OM-contact sites on ultrathin sections, inactive sperm taken directly from the testis and sea water-activated sperm in suspension were collected in cellulose capillary tubes (Hohenberg et al., 1994) and immediately high-pressure frozen (Müller and Moor, 1984), without prior chemical fixation. Subsequently, the samples were freeze-substituted and Epon embedded. Mitochondria prepared by these advanced electron microscopical (EM) preparation techniques revealed clear OM and IM aspects, as well as distinct cristae membranes (Figure 4). In contrast to the more or less ghost-like membrane relicts of mitochondria, seen after chemical fixation and conventional EM preparation techniques, which are still being pictured in textbooks as formal models of mitochondrial ultrastructure, the intermembrane space (IMS) between IM and OM was small (approximately 10 nm) and the matrix showed a rather compact electron dense appearance. This indicates that the membrane appearance

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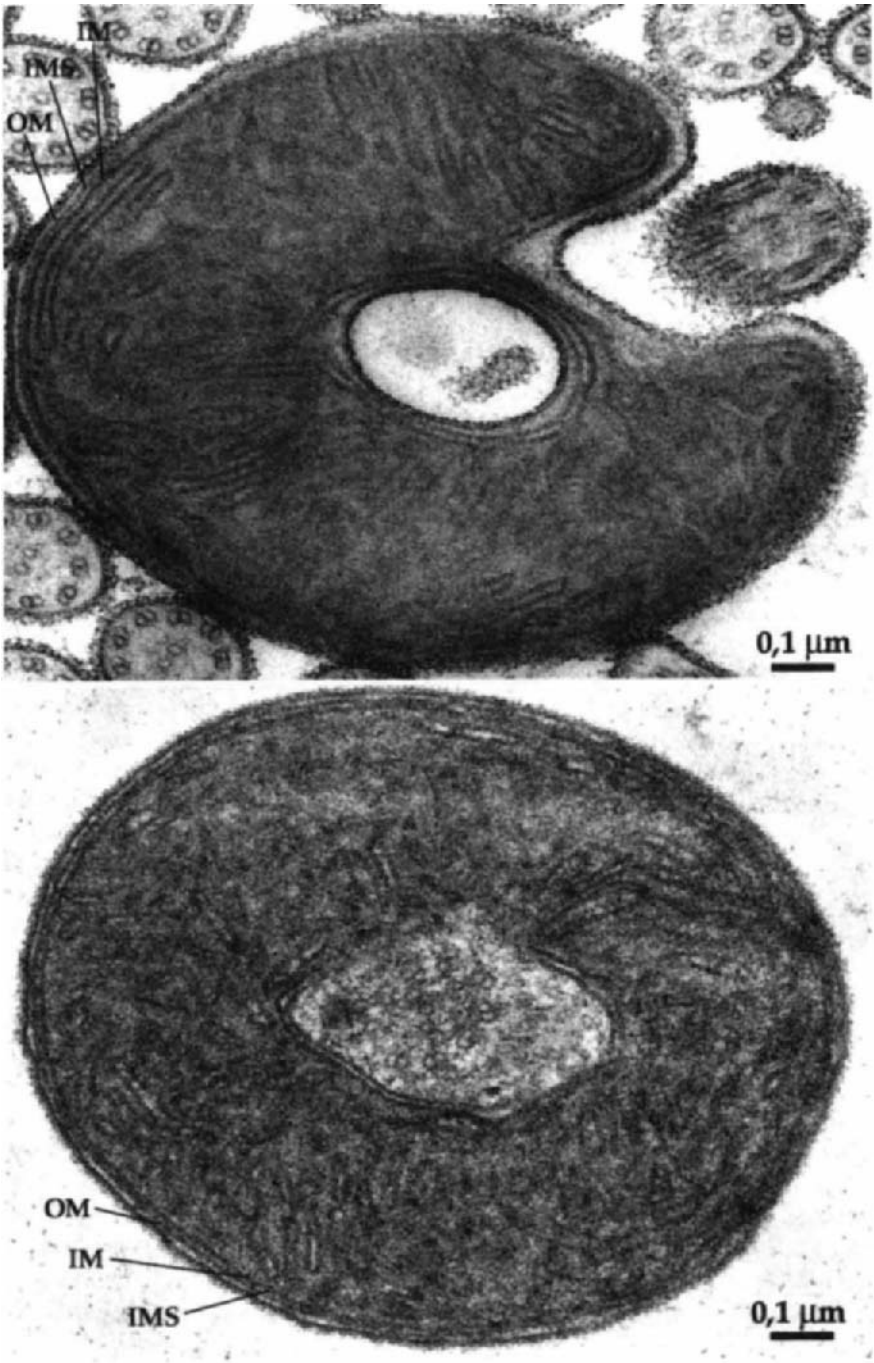
Figure 4. Mitochondrial ultrastructure and contact sites in activated and inactive sea urchin sperm. Electron micrograph of the single mitochondrion at the base of the sea urchin sperm head before and after sperm activation at high magnification. *Upper panel*: a mitochondrion of inactive sea urchin sperms, taken directly out from the testis. This cell suspension contained only quiescent, immotile spermatozoa. *Lower panel*: the same, but from sea water-activated sperm, containing only active, motile sperm, as checked by light microscopy and *in vitro* fertilization assays. The sperm plasma membrane on the very outside is ensheathing entirely the single mitochondrion. Note in both micrographs, the rather distinct but narrow separation of the inner mitochondrial membrane (IM) and outer mitochondrial membrane (OM) by a gap of approximately 10 nm, representing the intermembrane space (IMS), which, however is frequently interrupted by close appositions of IM and OM, presumably forming contact sites for energy export. However, no statistical difference in contact site frequency between inactive and activated sperm could be observed. Also note the rather unusual electron dense appearance of the mitochondrial matrix, seen in these mitochondria prepared according to advanced EM preparation techniques, comprising collection of sperm suspensions into cellulose capillary tubes (Hohenberg et al., 1994) and cryoimmobilization of the sperm by high-pressure freezing (Müller and Moor, 1984), followed by freeze-substitution and Epon embedding (Piendl, 1996).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is less disrupted and that less biological material is lost from within mitochondria by processing the sperm with advanced, compared to conventional, EM preparation methods (see Figure 4). However, a comparison of the frequency and measurements of clearly recognizable stretches of contact sites, where IM and OM were in close apposition, between inactive and activated sea urchin sperm (see Figure 4) revealed no obvious and statistically significant difference (not shown). Thus, we are led to assume that in sea urchin sperm, no major ultrastructural changes occur in mitochondria during cell activation and that the mitochondrial energy export contact sites are most likely already formed before sperm activation. In hindsight, this view is supported by the fact that, unlike with tissue from other species, Mi-CK of sea urchin sperm is rather difficult to be extracted quantitatively. Whereas the extraction of Mi-CK from vertebrate brain, skeletal, or cardiac muscle can be achieved by mitochondrial swelling in hypotonic buffer, followed by incubation in phosphate buffer at pH 8.0 or above (Schlegel et al., 1988b), relatively high concentrations of detergents are needed for extraction of Mi-CK in sea urchin sperm (Wyss et al., 1995). In addition, sea urchin sperm Mi-CK octamers, after extraction and isolation, turned out to be much more resistant to dissociation into dimers as compared to chicken sarcomeric Mi_b-CK (Wyss et al., 1995). This indicates that in the case of sea urchin sperm, Mi-CK is present only as octamers and in strong association with mitochondrial membranes. Thus, the dynamics of the dimer/octamer equilibrium or the membrane attachment/detachment properties proposed for other cellular systems (Wallimann et al., 1992; Wyss et al., 1992) seem to be absent in sea urchins. Concluding this section, the sea urchin sperm model, which was extremely useful for the demonstration of the importance of the CK system for energy shuttling, did not reveal new insight in terms of the possible dynamics of the formation of mitochondrial energy export contact sites, except that the latter may be already established in inactive spermatozoa of the sea urchin and thus be more static than anticipated from mitochondria in other cells and tissues from different species.

III. ROOSTER SPERMATOZOA

A. Structure of Rooster Spermatozoa

Spermatozoa from rooster, as well as from turkey and guinea fowl, share a structure that differs from sea urchin and mammals in two respects. First, the sperm heads are not round or ellipsoid but elongated with a width of only 0.5–0.7 μm and a length of 12.5 μm (see Figure 5; Lake et al., 1968; Thurston and Hess, 1987). Second, whereas sea urchins have only one single mitochondrion, fowl spermatozoa have 25–30 mitochondria that are helically arrayed around the distal centriole in the midpiece (Bakst and Howarth, 1975; Thurston and Hess, 1987). These mitochondria are densely packed as polygonal structures. Apart from those differences, fowl spermatozoa share a common structure, composed of a flagellum (70–90 μm long) with a 9+2 microtubular axonemal complex (see also Figure 7),

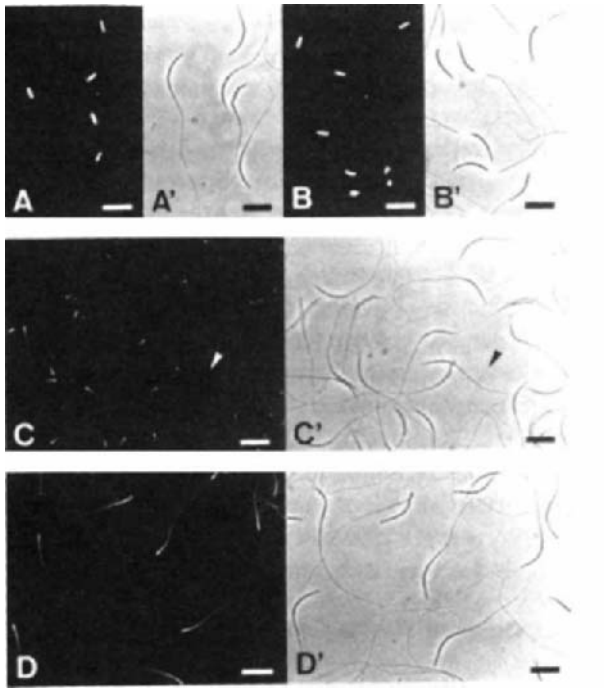

Figure 5. Localization of CK isoenzymes in rooster spermatozoa. Immunofluorescence microscopy of rooster spermatozoa using primary antibodies against Mi_b-CK (panel A and B), against Mi_a-CK (panel C), and against B-CK (panel D). The corresponding phase contrast images are shown for comparison in panels A', C', and D'. Panel B' shows a superimposed phase contrast and fluorescence picture. In panel A, B, and B' the monoclonal antibody 30a against Mi_b-CK at a dilution of 1:2, in panel C the polyclonal antibody against Mi_a-CK (which was cross-absorbed against Mi_b-CK) at a dilution of 1:300, and in panel D the polyclonal antibody against B-CK at a dilution of 1:200 was used. Note B-CK is most prominent in the proximal half of the tail. Some B-CK in the distal part is probably lost during permeabilization of the sperm by detergents needed for immunofluorescence staining. Also note that the polyclonal antibody against Mi_a-CK, also stains the very end of the sperm tail (marked by an arrowhead in panels C and C'). Scale bars = 10 μm. Similar localization of the rooster sperm CK isoenzymes, at higher resolution have been obtained by laser confocal microscopy (Kaldis et al., 1996a).

a midpiece with mitochondria and centrioles, and a head containing nucleus and acrosome (Lake et al., 1968; Tingari, 1973; Tingari and Lake, 1973; Bakst and Howarth, 1975; Lake, 1981; Thurston and Hess, 1987). Noteworthy is also the localization of the proximal centriole at the head-midpiece junction.

In mammals, spermatozoa need to be activated by incubation in the female tract in order to acquire the ability to fertilize an egg. This process is called capacitation and does not take place in fowl since their spermatozoa can fertilize eggs *in vitro* (Lake, 1984).

B. CK Activity in Fowl Spermatozoa

Activity of several enzymes including CK have been detected in rooster spermatozoa (Buckland, 1970). The presence of CK isoenzymes was later confirmed and the CK activity determined to be higher than in human spermatozoa (Wallimann et al., 1984, 1986). A detailed comparison of different species, revealed that birds (turkey, 350×), followed by echinoderms (sea urchin, 190×), annelids (lugworm, 32×), fish (carp, 20×), and mammals (rat/stallion, 1×) contain the highest specific CK activity (see Table 1; Kamp et al., 1996). However, these measurements should only be used for qualitative comparison, since in certain species it is difficult to quantitatively extract CK from spermatozoa. This is especially true for Mi-CK from sea urchin sperm (Wyss et al., 1995). In addition, CK from bull spermatozoa (see Section V) seem to be inhibited by a compound in the seminal plasma.

C. Rooster Seminal Plasma Contains Cytosolic Brain-type B-CK

As in human seminal plasma (Soufir, 1979; Asseo et al., 1981), B-CK was also detected in rooster seminal plasma as the sole CK isoenzyme (Kaldis et al., 1996a). To verify that the B-CK detected in seminal plasma was not due to leakage from the spermatozoa flagellum, rooster ejaculate, washed sperm, and seminal plasma were analyzed. The total ejaculate displayed a considerably higher B-CK signal than washed sperm and seminal plasma alone, indicating that a significant fraction of B-CK originates from the seminal plasma itself (Kaldis et al., 1996a). This is in contrast to earlier experiments, where no CK activity was found in rooster seminal plasma (Wallimann et al., 1986). There are at least three different possible reasons for this discrepancy: (1) different experimental procedure whereas higher centrifugal forces may damage sperm and lead to enhanced leakage of B-CK into the seminal plasma; (2) variation of the portion of immature sperm with attached cytoplasmic droplet that is released into the seminal plasma during centrifugation; and (3) variation of CK content in seminal plasma, as was shown for turkey (G. Kamp, unpublished data).

Thus, as proposed for some mammalian sperm (see Section V), the B-CK in rooster seminal plasma could also contribute to external energy supply to the spermatozoa, since addition of PCr to human ejaculate or washed sperm enhanced motility and velocity although to a lesser extent than ATP (Fakih et al., 1986; Wallimann et al., 1986). It remains to be investigated in which chemical form (PCr, ATP, or others) or through what kind of channels these substrates may be taken up by spermatozoa or whether these effects are mainly extracellular.

D. Three CK Isoenzymes Identified in Rooster Spermatozoa

Because fowl spermatozoa contain high levels of CK activity and highly specific antibodies against all chicken CK isoenzymes (Kaldis, 1994) were available, we focused on rooster spermatozoa to investigate the CK isoenzymes therein and their subcellular localization. B-CK was detected as the sole cytosolic CK isoenzyme (Wallimann et al., 1986; Kaldis et al., 1996a) and a Mi-CK was unambiguously identified as the sarcomeric mitochondrial isoenzyme (Mi_b-CK; Kaldis et al., 1996a). Surprisingly, an antibody against ubiquitous/brain mitochondrial CK (Mi_a-CK) that did not cross-react with Mi_b-CK stained a protein band with slightly different mobility than Mi_b-CK (Kaldis et al., 1996a). The pI of this protein was determined by 2D-gel electrophoresis as much more acidic than Mi_a-CK purified from chicken brain or Mi_b-CK from chicken heart. This sperm CK migrated even more acidic than B-CK and was therefore named as "Mi_a-CK variant" (Kaldis et al., 1996a). In addition, whereas B-CK and Mi_b-CK are easily recovered from spermatozoa by phosphate extraction following hypotonic shock, the "Mi_a-CK variant" could not be extracted using native conditions.

Several lines of evidence point to the fact that there are three different CK isoenzymes present in rooster spermatozoa. Two of the isoenzymes, B-CK and Mi_b-CK, are the same as can be found in other tissues of the chicken, although such a combination of those isoenzymes has not been found so far (see below). The third isoenzyme, was assigned as mitochondrial isoenzyme since in immunofluorescence experiments it stained mainly in the midpiece containing the mitochondria (see below, Figure 5C). Its characteristics resembled most closely Mi_a-CK (it was recognized by a specific antibody against Mi_a-CK) and was, therefore, termed "Mi_a-CK variant." At the same time, the "Mi_a-CK variant" differs substantially in pI when compared to genuine Mi_a-CK purified from chicken brain (Kaldis et al., 1996a) and seems to be a sperm-specific isoenzyme that has not been discovered in other tissues. Our results leave open whether the "Mi_a-CK variant" is a modified Mi_a-CK or actually a genuine new CK isoform. This question can only be resolved by protein sequencing, albeit many attempts have failed so far (P. Kaldis and T. Wallimann, unpublished). This is probably due to a blocked N-terminus as it was found for Mi-CK from sea urchin (Wyss et al., 1995). On the other hand, Northern blot analysis revealed that there is genuine Mi_a-CK mRNA present in rooster testis. The testis Mi_a-CK protein co-migrated on 2D-gels with genuine Mi_a-CK purified from chicken brain (M. Stolz and T. Wallimann unpublished). These results indicate that there might be genuine Mi_a-CK in sperm precursor cells. Therefore, the very acidic pI of the "Mi_a-CK variant" might be explained by posttranslational modification(s) of genuine Mi_a-CK. Though, many modifications like myristoylation do not change the pI of a protein (Quest and Shapiro, 1991), phosphorylation definitely can cause a shift to an acidic pI. Several CK isoenzymes (chicken M-, B-, Mi_b-CK, and rabbit B-CK) are capable of autophosphorylation (Hemmer et al., 1991). In addition, B-CK is a substrate for protein kinases, especially protein kinase C (Quest

et al., 1990; Chida et al., 1990a, 1990b). The nature of the possible posttranslational modification of the "Mi_a-CK variant," however, has not been worked out.

In conclusion, it is the first time that two different mitochondrial CK isoenzymes (Mi_b-CK and Mi_a-CK variant) were found to be coexpressed within one cell which may represent an adaptation to special requirements of sperm energetics. In addition, these results demonstrate the coexistence of brain-type cytosolic B-CK with the sarcomeric mitochondrial Mi_b-CK within one cell. In most other tissues/cells, B-CK is always coexpressed with Mi_a-CK or alternatively, M-CK is coexpressed with Mi_b-CK. A similar case was also found in Purkinje cells of the chicken brain where instead of the usual brain-type B-CK, the muscle-type M-CK (Hemmer et al., 1994) is coexpressed with a Mi_b-CK variant (Kaldis et al., 1996b).

E. Immunolocalization of the Rooster Sperm CK Isoenzymes

Immunofluorescence experiments revealed that B-CK is localized along the entire sperm tail and to a lesser extent everywhere where cytoplasm could be found (Figure 5D). The proximal half of the flagellum was much stronger stained than the distal half, indicating that during the permeabilization process needed for immunostainings, a significant part of B-CK was lost due to diffusion. Mi_b-CK, on the other hand, is exclusively located in the midpiece containing the mitochondria (Mi_b-CK, Figures 5A, 5B, and 5B′). Optical sectioning with confocal laser microscopy revealed that the midpiece is uniformly stained with the antibody against Mi_b-CK (Kaldis et al., 1996a).

The "Mi_a-CK variant" was localized at the head–midpiece junction where the proximal centriole is also located (Figure 5C). The midpiece was also stained by the antibody against Mi_a-CK in a non-uniform manner with the signal decreasing from the head–midpiece junction toward the end of the midpiece. In addition, the end of the flagellum (arrowhead in Figure 5C) and the acrosome region of the head (Kaldis et al., 1996a) were also stained. The localization of the "Mi_a-CK variant" is clearly distinct from Mi_b-CK that is exclusively found in the midpiece. To resolve the exact localization of the "Mi_a-CK variant" with higher precision, immunoelectron microscopy at an ultrastructural level will be needed.

F. Possible Functions of CK in Rooster Sperm

The subcellular compartmentation and distinct localization of the three CK isoenzymes in rooster spermatozoa indicates that they might have distinct functions in sperm energy metabolism. The finding that sperm contain B-CK was originally surprising (Wallimann et al., 1986) since one would have expected the presence of M-CK due to the similarity of the flagellar axoneme to the muscle fibers. In addition, M-CK was shown to associate with subcellular structures like the myofibrillar M-line or the sarcoplasmic reticulum, where M-CK is functionally coupled to the actomyosin ATPase (Wallimann et al., 1984b; Krause and Jacobus, 1992) and the Ca^{2+}-ATPase (Rossi et al., 1990; Korge et al., 1993; Korge and Campbell, 1994),

respectively. This leads to the suggestion that the B-CK found in sperm is associated with the axonemal dynein ATPase, although immunogold labeling of B-CK in the rooster sperm flagellum resulted only in weak signals, with some gold grains being definitely positioned over the axoneme (T. Piendl, unpublished).

Since both in sea urchin and rooster spermatozoa, inhibition of CK by DNFB results in loss of movement in the distal third of the flagellum (Tombes and Shapiro, 1985; Wallimann et al., 1986), it is very reasonable to speculate that B-CK is physically associated with the axonemal dynein ATPase. Maybe the sea urchin TCK (Wothe et al., 1990; Quest et al., 1992) reflects an adaptation to serve the axonemal dynein ATPase in a more efficient way.

The identification of a distinctly localized sperm specific "Mi_a-CK variant," albeit only representing a minor CK species in rooster sperm, as well as its coexpression with Mi_b-CK, came as a surprise. These results showing a rather unusual CK isoenzyme combination add further complexity to the CK system, possibly allowing for better adaptation to the special physiological situation in spermatozoa. This agrees with the finding that, for example, in sea urchins three different Mi-CK bands varying slightly in their apparent M_r can be detected (Tombes and Shapiro, 1985; Wyss et al., 1995).

Since Mi_b-CK co-localizes perfectly with the mitochondria in the midpiece, we believe that this isoenzyme is responsible for the traditional function assigned to it, that is, transphosphorylation of mitochondrial matrix generated ATP and export of phospho–creatine into the cytosol (Wyss et al., 1992). The "Mi_a-CK variant," on the other hand, is localized mainly to the head–midpiece junction exactly where the proximal centriole is found. In addition, the midpiece was stained in a non-uniform manner, suggesting that if the "Mi_a-CK variant" co-localizes with the proximal (and distal) centriole, the non-uniform staining could be due to masking of the centrioles by the mitochondria. In other words, the antibodies may be able to reach the centrioles only at gaps between neighboring mitochondria. At this defined location, the "Mi_a-CK variant" could be directly associated with the ATPases of the centrioles and serve special requirements of the centriole during the formation of the axoneme, as well as for the stabilization of the centrioles and the proximal ends of the microtubules. The same could hold true for the small amount of "Mi_a-CK variant" localized specifically at the very tip of the sperm tail, where the enzyme in combination with a guanylate kinase could locally maintain high levels of GTP to stabilize the distal end's microtubules in the axoneme.

In addition, the specific staining of the "Mi_a-CK variant" in the acrosome region indicates an involvement of CK for energy supply during the acrosome reaction. Interestingly, the antibody against B-CK also stained the acrosome, though at a clearly different region compared to the "Mi_a-CK variant" (Kaldis et al., 1996a).

Finally, labeling of rooster spermatozoa with ^{32}P-ATP resulted in a strong band at 43 kDa, but only if the sperm were motile (Ashizawa et al., 1992). We could imagine that this 43 kDa protein is CK, since inhibition of CK in rooster sperm leads to a loss of flagellar movement (Wallimann et al., 1986).

IV. A TESTIS- AND SPERM-SPECIFIC CK ISOENZYME IN TROUT

Sea urchin sperm tail CK (TCK) could be considered entirely as a sperm-specific CK isoenzyme. In higher vertebrates, however, the first evidence for a testis and sperm-specific CK isoform came from experiments with trout. In this fish, a novel CK cDNA whose transcript showed enhanced testicular expression was identified (Garber et al., 1990). The predicted N-terminal amino acid sequence for this CK subunit shows a novel coding region which has now been confirmed by purification and N-terminal sequencing of the protein (Saudrais et al., 1996). In addition, *in situ* hybridization and immunohistochemical staining with an antibody against sperm CK (s-CK) revealed the presence of its transcripts in primary and secondary spermatocytes, as well as in early spermatids, whereas the protein was detected in late spermatids and spermatozoa only. These results demonstrate that this s-CK isoenzyme is a germ cell specific protein, the transcription of which starts in meiotic germ cells, while its translation starts in late spermatids (Saudrais et al., 1996). Unfortunately, nothing is known yet about the subcellular localization of this sperm CK within trout spermatozoa.

The Mi_a-CK variant discovered in rooster sperm, although it is a minor CK species, could also be termed as sperm-specific, since so far it has not been found in any other tissue of the same species and since it shows a distinct localization in the rooster sperm cell (see above). Based on these data, CK could thus be considered as an enzyme that joins as a late member those isoenzyme families with sperm-specific isoforms.

Sperm-specific isoenzymes have also been found in other enzyme families including hexokinase from mouse (Kalab et al., 1994) and arginine kinase from horseshoe crab (Strong and Ellington, 1993), or sperm-specific lactic dehydrogenase, called LDHX or $LDHC_4$, discovered in the 1960s (Zinkham et al., 1964), being the first classical example of this case (Pan et al., 1983). These sperm- specific isoenzymes may differ sufficiently in their atomic structure, especially around the catalytic site, as well as in their catalytic properties and mechanisms from those isoforms expressed in somatic cells. Therefore, these sperm-specific isoenzymes could be targets for anticonceptive strategies in the future.

V. CREATINE KINASE IN MAMMALIAN SPERM AND SEMINAL FLUID

A comparative view of CK in species with differences in sperm structure, energy metabolism, and the mode of reproduction is a valuable approach for understanding the differences in CK expression and its adaptation to sperm function. As mentioned above, CK isoenzymes and CK function of sea urchins and rooster spermatozoa are well established and there is good evidence for a CK shuttle in these specialized sperm cells. This is corroborated by physiological investigations indicating a clear

preference for mitochondrial ATP production. However, the situation in mammalian spermatozoa is quite different and the species-specific expression of CK does not allow a general scheme for CK function. The generally much lower CK activities found in mammalian sperm (Table 1) suggest either a moderate function for the PCr shuttle in combination with an alternative route, for example via glycolysis or the adenylate kinase system, to supply distal dynein ATPases or other functions (e.g., external ATP delivery), which are discussed in the following chapter.

A. Human Sperm: CK Isoenzyme Ratios as an Indicator for Infertility

Investigations on CK in human ejaculates led to the identification of CK isoenzymes in the spermatozoa and also in the seminal plasma (Soufir, 1979; Asseo et al., 1981; Wallimann et al., 1984a). Seminal plasma CK was identified as B-CK (Wallimann et al., 1986), which may be secreted by the accessory glands, the prostate (Kavanagh and Darby, 1983) or the seminal vesicles (Soufir, 1979). The appearance of CK in the seminal plasma is accompanied by the presence of substrates, mainly Cr, which has led to the assumption of an external energy supply for human spermatozoa through the CK reaction (Wallimann et al., 1986; Lee et al., 1988). This was supported by the detection of substrates (PCr, Cr, ATP, ADP) in the fluids of seminal vesicle glands or of high Cr levels in the testis (Lee et al., 1988), whereas Arrata et al. (1978) could not detect PCr in human semen by ^{31}P-NMR spectroscopy. Surprisingly, PCr supplied to isolated human sperm seems to enhance sperm motility and velocity (Fakih et al., 1986), but it is still unknown whether this external PCr can be taken up by intact sperm and thus be utilized intracellularly as an immediate energy source for ATP regeneration, or whether it is metabolized by extracellular CK. In the latter case, PCr would mediate its positive effect on sperm motility by an indirect mechanism. For example, extracellular protein kinases ("ectokinases") have been found in some mammalian semen (Lee et al., 1988) which are possibly involved in the phosphorylation of proteins of the plasma membrane surface during sperm maturation. This process requires external ATP which could theoretically be delivered by CK from the epithelial cells of the epididymis to the sperm surface.

Human spermatozoa showed two CK isoforms which, similarly to the situation in rooster sperm (Wallimann et al., 1986; Kaldis et al., 1996a; see above), have been immunologically identified as B-CK, representing the major form, and mitochondrial Mi-CK as the minor form (Wallimann et al., 1986). While the presence of B-CK in human spermatozoa is not disputed, the identity of another CK isoenzyme, based on its electrophoretic mobility, was reported to be similar to that of muscle-type cytosolic M-CK (Huszar and Vigue, 1990). However, in the same paper it was demonstrated that the two CK isoforms are different in structure. Therefore, this CK isoenzyme will be referred to in the following as M(sp)-CK. The hybridization of human sperm B-CK and M(sp)-CK did not result in the formation of MB-CK,

whereas after the replacement of sperm M(sp)-CK with the muscle-type M-CK the MB-CK heterodimer was found (Huszar and Vigue, 1990). In most recent work, the sperm M(sp)-CK in men was further characterized. Preliminary results show that the apparent Mr of the enzyme is 62 kDa, and the active form of the enzyme is the dimer of about 130 kDa, which elutes earlier from Sephadex G-150 than the 80 kDa B-CK dimer (Huszar and Vigue, 1997a). Thus, it is likely that there are three different types of CK isoforms in human spermatozoa: B-CK, Mi-CK, and the yet unclassified sperm CK with muscle-type CK electrophoretic characteristics [M(sp)-CK].

However, regardless of the identity of the sperm M(sp)-CK isoform, Huszar and Vigue (1990) reported men-to-men differences in sperm CK activity and in the proportion of the B-CK and M(sp)-CK isoforms, the "M(sp)-CK ratio" [%M(sp)-CK/{M(sp)-CK+B-CK}]. There was a fourfold higher CK activity and a fourfold lower "M(sp)-CK ratio" in oligospermic men (<20 million sperm/ml), who have a higher proportion of immature sperm than that of normospermic men (Huszar and Vigue, 1990). More importantly, there was a correlation ($r = -0.69$, and $P > 0.001$, $N = 159$ men) between the CK activity and "M(sp)-CK ratio" parameters (Huszar and Vigue, 1990). Based on these data, and on the demonstration of the relationship between the increased CK activity and cytoplasmic retention by CK immunocytochemistry in individual sperm, Huszar et al. suggested that the two events are developmentally related. The hypothesis suggested that the high CK activity in sperm is due to cytoplasmic retention because these sperm did not complete cytoplasmic extrusion and thus are immature and also deficient in functions such as fertilization potential. Further, the relationship between the CK activity and the "M(sp)-CK ratio" suggests that the cytoplasmic extrusion and the commencement of the M(sp)-CK biosynthesis are simultaneous events during spermiogenesis (Huszar and Vigue, 1990; 1993; Lalwani et al., 1996).

This resembles the sequential expression of CK isoforms during differentiation of muscle cells (Cohen et al., 1978; Perriard et al., 1978). In fetal skeletal and cardiac muscle, the B-CK isoform is predominantly present and the M-type as well as the mitochondrial isoenzyme are synthesized later in early development (Hoerter et al., 1991). Mitochondrial Mi-CK is also developmentally expressed even later (reviewed in Wyss et al., 1992). Mi-CK is clearly present in human spermatozoa as demonstrated by immunological means (Wallimann et al., 1986) and by labeling human sperm mitochondria with antibodies against Mi-CK (T. W., unpublished result). Based on the presence of Mi-CK in rooster and human sperm, the possibility was entertained that the unidentified M(sp)-CK in human sperm is an Mi-CK. However, based on the newer information on the molecular weight differences and due to the lack of reaction of the human sperm M(sp)-CK with antibodies against Mi-CK (Huszar and Wallimann, unpublished result), this possibility seems less likely, and the data suggest that M(sp)-CK may be a true human sperm-specific CK isoform. However, a detailed characterization of this possible human sperm-specific CK isoform is still needed.

In subsequent work at the Huszar laboratory, there was the following evidence that the plasma membrane of human sperm is also remodeled during spermiogenesis: (a) sperm with cytoplasmic retention do not recognize or bind to the zona of oocytes, (b) there was a relationship demonstrated between CK activity and the rate of lipid peroxidation in human sperm samples ($P < 0.001$, $N = 143$; Lalwani et al., 1996) and (c) there was a close relationship ($r = 0.78$ and -0.8, $P < 0.001$, $N = 70$) between CK activity or "M(sp)-CK ratio" vs. the concentration of sperm plasma membrane β1,4-galactosyltransferase, an enzyme which facilitates sperm–zona interaction in mice (Huszar et al., 1997b).

The relationship demonstrated between the CK biochemical markers and cytoplasmic extrusion in spermiogenesis (Huszar and Vigue, 1990, 1993), which is further supported by recent studies (Gomez et al., 1996), corresponds well with the structural evidence. Mammalian spermatozoa released from the testes pass through a complex maturation process in the epididymis, an event that is characterized by a loss of cytoplasma and changes in the protein composition on the outer plasma membrane (Philippe, 1974). Figure 6 shows migration of a typical cytoplasmic droplet along the midpiece and its release from the posterior midpiece. This process removes most of the cytoplasm from the mitochondrial sheath including membrane stacks of the endoplasmic reticulum and ribosomes and consequently stops any protein synthesis.

During this process of "shedding," there will be an increase in the relative ratio of M(sp)-CK (whether sperm M(sp)-CK or Mi-CK) versus B-CK, the ratio that predicted both occurrence of pregnancies and infertility in a study of IUI and IVF couples (Huszar and Vigue, 1993). Indeed, in the most mature human sperm population with the least amount of retained cytoplasm, which may be recovered from 80% Percoll pellets, as much as 60 to 80% of the constituent CK is the M(sp)-CK isoform (Huszar and Vigue, 1993). These studies with CK activity and the M(sp)-CK isoform were useful in establishing the relationship between sperm maturity and sperm function and in developing objective biochemical markers of male fertility.

From a clinical perspective, a reinforcement of the CK system in human testis and/or spermatozoa (e.g. by supplementation with creatine or creatine analogues; Fakih et al. 1986) may serve as adjuvant therapy for certain types of male infertility. In addition, creatine or creatine analogues given to the medium may increase the efficiency of *in vitro* fertilizations, as well as support early embryogenesis. On the other hand, sperm CK may represent a valuable target for anticonception strategies, using CK substrate analogues or CK inhibitors to interfere or inhibit, respectively, with sperm energetics and thus sperm motility.

B. Bull Sperm: External CK and CK Inhibition in Epididymis Fluid

In contrast to man, bull spermatozoa obtained from ejaculates do not show CK activities (Table 1) and no PCr has been detected biochemically (Brooks, 1971) or by ^{31}P-NMR therein (Robitaille et al., 1987). However, recent analysis of CK

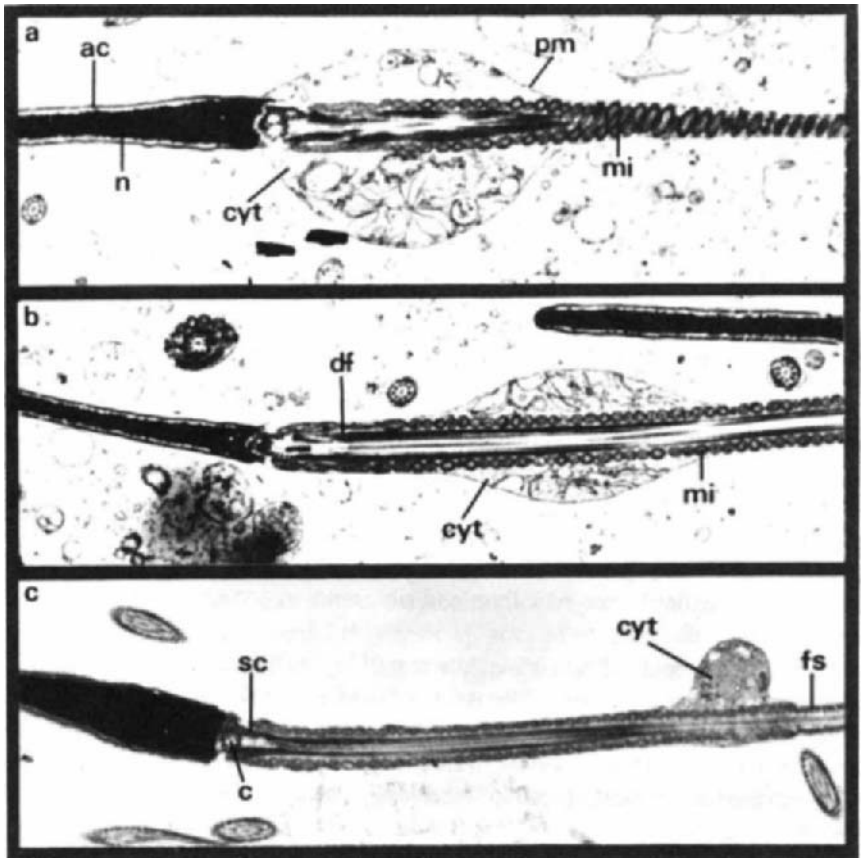

Figure 6. Migration of cytoplasmic droplets along the midpiece of bull spermatozoa (Bone and Kamp, unpublished). Transmission electron micrographs reveal residual cytoplasm of the midpiece with large numbers of vesicles which is cast off from the neck (a; 9,000×) toward the principal piece (b; 12,100×). The such generated droplet is cut off where the midpiece adjoins the principal piece (c; 9,100×). ac: acrosome; c: centriole; cyt: cytoplasma; df: dense fibers; fs: fibrous sheath; mi: mitochondria; pm: plasma membrane; sc: striated column.

activity of Ficoll-washed bull spermatozoa obtained from different parts of the epididymis clearly revealed CK activities with a maximum in the middle piece (corpus) of the epididymis (Bone and Kamp, unpublished data). The epididymal fluid also contains high levels of CK activity. Both sperm and external fluid CK activities completely disappear in the semen, probably as a result of a very potent inhibitor secreted from the accessory glands. This inhibitor affects bull semen CK irreversibly and also CK of other cells as well as other kinases such as hexokinase.

From control experiments, we know that neither NADH oxidases nor ATPases are responsible for the inhibition of the kinase activities measured in a coupled enzymatic assay using NAD(P)H as indicators. A proteolytic effect can also be excluded. From no other semen (rat, stallion, boar, and man) was a similar effect observed, and the nature and physiological function of this inhibition is unknown.

The fact that washed bull spermatozoa of the epididymis show CK activity, but that the activity is inhibited in the ejaculate by an external inhibitor, indicates that CK is localized at the outside of the plasma membrane. A penetration of the inhibitor into the intracellular space of spermatozoa is unlikely because activities of intracellular hexokinase or pyruvate kinase are detectable in washed spermatozoa. The situation in bull spermatozoa is not consistent with the general consideration of guanidino kinase function as an intracellular ATP regenerating system for motility (Schoff et al., 1989). This conclusion is reinforced by the analysis of bull sperm metabolites revealing no PCr and no Cr but normal nucleotide levels in washed spermatozoa (Bone and Kamp, unpublished data). Cr, however, was found in bull seminal plasma (Mann and Lutwak-Mann, 1981) and could be a substrate of external CK in the epididymis.

C. Boar Sperm: The Reason for Aerobic Lactate Production

Like that of bulls, boar spermatozoa obtained from semen did not show CK activity (Table 1) and correspondingly ^{31}P-NMR spectra did not reveal a PCr resonance either in intact cells or in sperm extracts (Robitaille et al., 1987). The lack of PCr (no intracellular ATP buffer) probably explains the high fluctuations of the intracellular ATP level in boar spermatozoa (11.3 to 2.8 nmol/10^8 sperm) which depend on beat frequency and the availability of external substrates (Kamp, unpublished data). Like other mammals, boar spermatozoa preferentially produce ATP from carbohydrate (glucose or fructose) fermentation to lactate even under aerobic conditions (for review see Mann and Lutwak-Mann, 1981). Intracellular and extracellular lactate accumulation may be caused by the compartmentation of mitochondria in the midpiece (Figure 7e) and of the glycolytic pathway in the principal piece. Previous studies on the compartmentation of glycolytic enzymes in these sperm revealed association of flux-controlling enzymes, such as hexokinase and phosphofructokinase, with cell structures (Harrison, 1971), but in the case of glyceraldehyde 3-phosphate dehydrogenase, a specific attachment of this enzyme to the fibrous sheath being a typical structure of the principal piece in mammalian spermatozoa (Figures 7E and 7F) has been recently demonstrated by immunogold labeling (Westhoff and Kamp, 1997). This first result supports the assumption of a preferential compartmentation of glycolysis in the principal piece of boar and probably also in other mammalian sperm. This suggests a job sharing in ATP production between mitochondria in the midpiece and glycolysis in the principal piece. Hence energy transfer from the mitochondria to the distal dynein ATPases would be replaced by glycolytic ATP production in the principal piece.

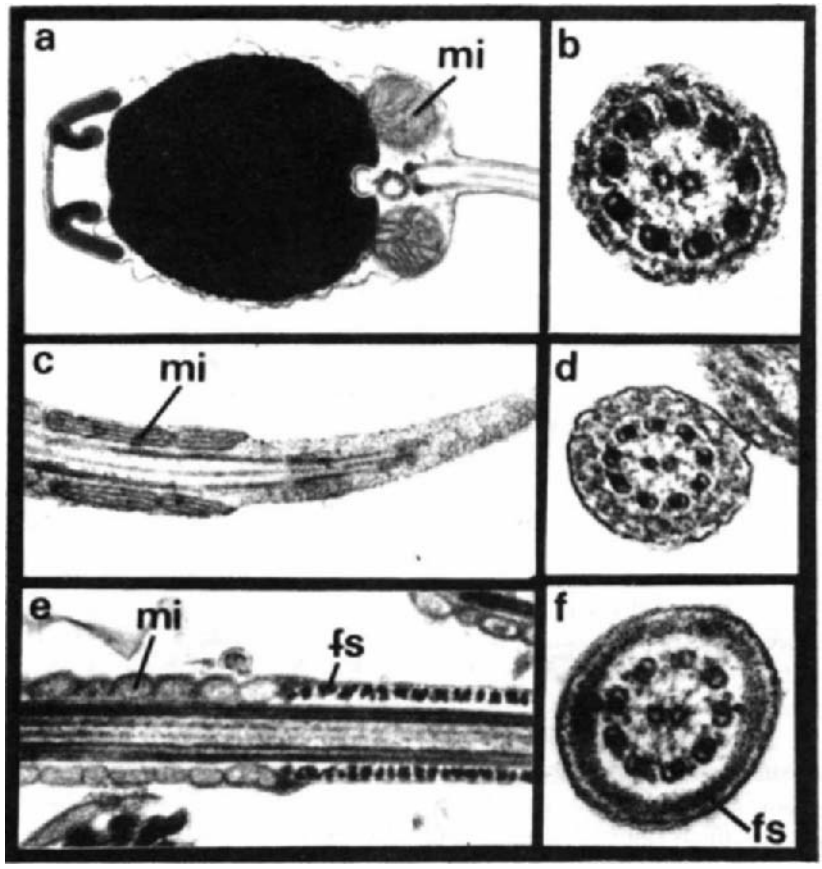

Figure 7. Variation in the structure of spermatozoa among different species. Transmission electron micrographs of lugworm *Arenicola marina* spermatozoa (a; 22,500× and b; 135,000×) were taken from Kamp et al. (1995), that of turkey (c; 25,000× and d; 76,000×) and boar (e; 36,000× and f: 114,000×) spermatozoa from Kamp et al. (1996). Mitochondria in lugworm spermatozoa are located just behind the nucleus (a) which is typical for the so called "primitive" spermatozoa. The transverse sections (b), (d), and (f) show the typical 9+2 arrangement of the microtubuli. The longitudinal sections of turkey (c) and boar (e) represent the connection between the midpiece, containing mitochondria (mi); and the principal piece. (fs) is the fibrous sheath which is a typical rather electron dense compact structure in mammalian spermatozoa.

In these sperm, lactate permeates the membrane and accumulates in the extracellular space but it can also be transferred to the mitochondria for further oxidation. Correspondingly, mammalian sperm mitochondria contain a specific lactate dehydrogenase which delivers the substrate pyruvate and reduces NAD (Baccetti et al., 1975; Gallina et al., 1994). It is obvious that the transfer of lactate instead of pyruvate and NADH has a great advantage in the flagellum because the concentration of lactate is much higher than that of NADH and, therefore, improves the diffusion over the long distance to mitochondria.

It seems likely that the compartmentation of energy metabolism proposed for boar spermatozoa can also be transferred to spermatozoa of other mammals because energy metabolism (Mann and Lutwak-Mann, 1981), cell structure (Philippe, 1974), and the maximum enzyme activities (Table 1), as well as some properties of glycolytic enzymes (Kamp, unpublished) are very similar.

D. Sperm of Other Mammals: Balance between Fermentation and Oxidation

CK has been found in sperm from stallion, rat (Table 1), and mice (Steeghs et al., 1995). In mice, besides cytosolic B-CK, the ubiquitous mitochondrial CK isoform, Mi_a-CK, has been detected (Steeghs et al., 1995). It may be possible that the extent of CK and especially of Mi-CK indicates a shift in the ATP production from fermentation toward mitochondrial oxidation assuming that a PCr shuttle improves the diffusion of mitochondrial energy-rich phosphate through the flagellum. A certain plasticity in the utilization of ATP producing pathways may also explain the results obtained with transgenic mice mutants lacking Mi_a-CK, surviving without significant effect on sperm motility and fertility (Steeghs et al., 1995). Probably, transgenic mice produce spermatozoa which are adapted to the lack of Mi-CK either by shifting ATP production toward fermentation or by compensatory utilization of alternative systems.

The fact that fermentation plays an important role in the energy metabolism of mammalian spermatozoa does not implicate an anoxic tolerance. Mitochondrial oxidation is certainly essential for spermatozoa of most species because anoxia or poisoning of the respiratory chain by cyanide reduced beat frequency (for review see Mann and Lutwak-Mann, 1981). Moderate hypoxia down to 5% of normoxic oxygen tension, however, does not influence the rate of oxygen consumption. This surprising finding can probably be explained by the high oxygen affinity of the cytochrome oxidase and the low diffusion distance of oxygen between the extracellular space and the mitochondria. This may enable mammalian spermatozoa to perform normal mitochondrial activity in the female tract, an environment which is certainly not anoxic but hypoxic under normal physiological conditions (for review see Mann and Lutwak-Mann, 1981).

E. Energy Channeling through the Adenylate Kinase System

The adenylate kinase (AK) system has been proposed as an alternative AK shuttle in spermatozoa lacking CK (Schoff et al., 1989). In this case, Cr is replaced by AMP as the mitochondrial acceptor of energy-rich phosphate, and the CK isoenzymes by the corresponding cytosolic and mitochondrial AK isoforms (Tanabe et al., 1993). A mitochondrial AK has also been demonstrated in muscle tissues (e.g., the rat heart; Gellerich et al., 1987). By this pathway, AMP would be successively phosphorylated to ADP plus ATP by an interplay of the mitochondrial AK with the mitochondrial ATP synthase. The ATP thus formed would return to the dynein ATPases where it is hydrolyzed to ADP which can subsequently be used to phosphorylate a second ADP through cytoplasmic AK (2 ADP $\rightarrow$ ATP + AMP). The transfer of two energy-rich phosphates per molecule AMP is certainly an advantage of the AK shuttle, but the low cytoplasmic concentration of AMP compared with Cr may cast some doubt on the physiological significance of an AK shuttle that would have to cover the long distances between mitochondria and distal dynein ATPases (>20 μm). Otherwise, the AK shuttle may channel energy between ATP producing and consuming reactions in the cytoplasm (e.g., pyruvate kinase and phosphofructokinase or ATPases; Zeleznikar et al., 1995) or in the mitochondrial intermembrane space (e.g., translocator and hexokinase), thus preventing an accumulation of cytosolic ADP in the flagella which may be important for the thermodynamic efficiency of the various ATP producing and consuming reactions. An intricate interrelationship, as well as a plasticity between the CK and the AK system has recently been elegantly described for diaphragm muscle containing both cytosolic and mitochondrial isoforms of the respective enzymes. In this muscle, upon progressive CK inhibition with DNFB, the net CK catalyzed flux decreases, but the AK catalyzed flux increases reciprocally to compensate for the defect in CK function (Dzeja et al., 1996).

VI. EVOLUTIONARY ASPECTS OF GUANIDINO KINASES AND ADAPTATION OF SPERM ENERGY METABOLISM IN DIFFERENT SPECIES

Studies on spermatozoa of invertebrates have clearly revealed that CK is much more widespread in the animal kingdom than suggested previously (van Thoai and Robin, 1979). Even very primitive animals like sponges contain PCr (Roche and Robin, 1954). These examples clearly demonstrate that CK is not confined to vertebrates or to deuterostomes. The latter include the echinoderms which are well known for their guanidino compound heterogeneity and their preference for the CK system in sea urchin sperm (Moreland et al., 1967). As known so far, CK is also the guanidino kinase of annelids (primitive protostomes), the phylum in which seven different guanidino compounds and guanidino kinases were discovered (van Thoai and Robin, 1979; Kamp, 1989). A cytoplasmic and mitochondrial CK have been

discovered in sperm of the marine polychaete *Arenicola marina* (Kamp et al., 1995). On the other hand, the leech, *Hirudo medicinalis*, is a curious example for an animal living without any guanidino compound as demonstrated by *in vivo* ^{31}P-NMR (Schmidt and Kamp, 1996). During the further evolution of protostomes, arginine kinase (ArgK) is used instead of CK. PCr or CK activities were not detected in mollusks or arthropods though many tissues and sperm were investigated. This is in contrast to the evolution of deuterostomes which is accompanied by the disappearance of ArgK in vertebrates.

Ellington (1989) compared the different guanidino kinase systems discovered in the animal kingdom and has clearly shown that the CK system, due to its being thermodynamically more unstable PCr, is advantageous for buffering of cellular ATP at much higher ATP/ADP ratios. A disadvantage of PCr may be the relatively low half-life time of Cr because it is converted spontaneously by a non-enzymatic reaction to creatinine which permeates through cell membranes and is excreted with urine. About 50% of total body Cr is replaced in rats within 29 days (Bloch et al., 1941). A big advantage, however, of creatine, compared to arginin, is the fact that the former is disconnected from basic metabolism and, unlike arginine, does not interfere with the reactions of household enzymes.

Kamp et al. (1996) quantified CK activities in the sperm of various vertebrates and invertebrates and compared the catalytic capacity with those of typical marker enzymes of other important energetic pathways (Table 1). As mentioned above mammalian sperm energy metabolism is characterized by low CK and low HOADH activities (B-oxidation), while GAPDH, LDH, and CS activities are considerably higher compared to those of lower vertebrate or invertebrate sperm. This corresponds with the preference of mammalian spermatozoa for carbohydrate utilization, oxidation, or both together and is in good agreement with previous studies (for review see Mann and Lutwak-Mann, 1981).

In contrast, sea urchin spermatozoa revealed a high capacity for fatty acid oxidation and display high CK activities. This correlation confirms the role of a CK shuttle between mitochondria and distal dynein ATPases as mentioned above. Sea urchin spermatozoa may not be exposed to anoxia during either spermatogenesis or during swimming in the free water (external fertilization) and, therefore, do not require oxygen-independent ATP-producing pathways (glycolysis). Fat is an optimal fuel for long-lasting motility and allows sperm to be motile over a long period of time.

The lugworm *Arenicola marina*, however, lives in the mud of marine intertidal zones where it is exposed to prolonged periods of anoxia during low tide. The animal is well adapted to anoxia and consequently its sperm (Figures 7a and 7b) also revealed high capacities of fermentation (Elsing et al., 1994). During external fertilization internal stores of both carbohydrates and fat are available as fuels and enable these sperm to swim for several hours. An estimation of diffusion fluxes of metabolites involved in the PCr shuttle has revealed that Cr flux is several orders of magnitude higher than that of cytoplasmic ADP and probably ensures an

unlimited transport of the mitochondrial energy-rich phosphate to the distal dynein ATPases (Figure 1; Kamp et al., 1995).

Fertilization also externally occurs with carp, but males spawn close to the eggs shortly after their release on the bottom of a lake by the female. Correspondingly, the motility of carp spermatozoa is maintained only for a few minutes. In these sperm, ^{31}P-NMR spectroscopy has revealed a good correlation between the time of exhaustion of sperm motility, the depletion of the PCr pool, and a significant decrease in the ATP level (Büsselmann, unpublished data). This observation suggests that PCr functions as a short-term temporal ATP buffer in carp spermatozoa which is not immediately replenished, for example, by substrate oxidation in mitochondria. This is not surprising because the mitochondria of carp sperm are completely separated from the flagellum (Kamp et al., 1996).

Turkey spermatozoa (Figures 7c and 7d) are motile for several hours without external carbohydrates but intracellular glycogen stores are not available. Fat might be the most important fuel despite the relatively low HOADH activity. Although the PCr pool is rapidly reduced, the motility appears to be unaffected (Kamp et al., 1996). The CK activity is extremely high in turkey spermatozoa which could be related to the fact that they are not only preferentially fueled by fat oxidation but also show a relatively long flagellum in relation to the midpiece. These data present convincing evidence that sperm cells, even though they all look similar at first glance and have the same function, are extremely adapted in fine-structural details and metabolic out-fits to guarantee optimal function under the different environmental conditions where fertilization takes place.

VII. COMPARISON

From the data presented above, it becomes clear that no universal role can be assigned to CK for sperm function. In some spermatozoa, as in those from sea urchins, CK seems absolutely required for cell motility. In other cases CK may be less important for this function, but could in addition participate in the energetics of the acrosomal reaction and/or the stabilization of the microtubules in the axoneme. On the other hand, it is less important or even absent in other specialized cases, as for certain mammalian sperm cells, for example, boar and bull spermatozoa, which seem to derive most of their energy from external sources and are adapted to metabolize these external substrates via corresponding sets of glycolytic enzymes present in these specialized cells.

The question of whether CK belongs, at least in certain cases, to those sperm proteins which are either participating directly in egg activation, as certain ion channels may do (Lievano et al., 1996), or which are needed for early egg development, as has recently been shown to be the case for the SPE-11 gene product in *C. elegans* (Browning and Strome, 1996), remains to be answered in the future. A suitable approach would be the generation of double knock-out mice lacking both isoenzymes, B-CK as well as ubiquitous Mi-CK found in mouse spermatozoa

(Steeghs et al., 1995). The single deletion of the mitochondrial CK in mouse sperm has proven not to adversely affect fertility of transgenic mice (Steeghs et al., 1995). However, no viable double knock-outs of B-CK plus Mi-CK have been produced so far. Therefore, it is too early to dismiss an important role of CK for sperm function in the mouse (Wallimann, 1994).

The highly specialized sea urchin sperm, being dependent on PCr and CK, provided a very useful *in vivo* model to demonstrate unambiguously the energy transport function of the PCr/CK system to compensate for diffusional limitations of ATP, and much more so of ADP, in these highly polar cells with a long tail and correspondingly long diffusion distances (see Figure 1).

ABBREVIATIONS

AK, adenylate kinase; ArgK, arginine kinase; B-CK, cytosolic brain-type CK isoenzyme; CK, creatine kinase (EC 2.7.3.2); Cr, creatine; CS, citrate synthase (EC 4.1.3.7); DNFB, 2,4-dinitro-1-fluorobenzene; EM, electron microscopy; FCCP, trifluoro methoxyphenyl hydrazone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); HOADH, 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35); IM, inner mitochondrial membrane; IMS, intermembrane space; LDH, lactate dehydrogenase (EC 1.1.1.28); M-CK, cytosolic muscle-type CK; Mi-CK, mitochondrial CK isoenzymes; Mi_a-CK, ubiquitous Mi-CK isoenzyme, predominant in brain; Mi_b-CK, sarcomeric Mi-CK isoenzyme, predominant in sarcomeric muscle; OM, outer mitochondrial membrane; PCr, phospho–creatine; pI, isoelectric point; SDS, sodium dodecyl sulfate; s-CK/TCK1, sperm/testis CK from trout; TCK, flagellar tail CK from sea urchin.

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