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Activation of sea-urchin sperm motility is accompanied by an increase in the creatine kinase exchange flux

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The kinetics of the creatine kinase (CK) reaction were studied in suspensions of quiescent and active, intact sea-urchin spermatozoa in artificial seawater, using ^{31}P-NMR magnetization transfer. In inactive sperm, no CK-mediated exchange flux was detected, whereas in activated motile sperm, the forward pseudo-first-order rate constant was $0.13 \pm 0.04\ s^{-1}$ at 10 °C, corresponding to a steady-state CK flux of $3.1 \pm 0.5\ mM \cdot s^{-1}$. Intracellular pH shifted from 6.6 ± 0.1 to 7.6 ± 0.1 upon activation. The phosphocreatine (PCr)/ATP and PCr/P_i ratios were only marginally reduced in activated sperm, whereas the estimated cytosolic free ADP concentration increased remarkably from 9 μM in quiescent, to 114 μM in activated spermatozoa. The elevation of CK flux upon sperm activation is discussed in the light of the proposition that in sea-urchin spermatozoa, which are fuelled entirely by oxidative phosphorylation, high-energy phosphate transport is mediated by a 'CK/PCr shuttle'.

INTRODUCTION

Creatine kinase (CK) catalyses the reversible exchange of a high-energy phosphoryl group between ATP and phosphocreatine (PCr): $PCr^{2-} + MgADP^{-} + H^{+} \leftrightarrow$ creatine(Cr) $+ MgATP^{2-}$. The enzyme is abundant in mammalian muscle and brain [1]. Interestingly, high specific CK activities and high levels of PCr have also been observed in sea-urchin and fowl spermatozoa, but to a much lesser extent in mammalian spermatozoa [2–4]. In most cells, cytosolic CK isoenzymes are co-expressed with a distinct mitochondrial CK isoform. In sea urchin, as well as rooster, spermatozoan mitochondrial CK appears as an octameric isoenzyme that is located within the mitochondrial intermembrane space (mitochondrial isoenzyme of creatine kinase: Mi-CK) [5,6]. An additional Mi-CK variant has recently been identified in rooster spermatozoa, and was localized at the mitochondrial/nuclear boundary, at the base of the acrosome, as well as at the very tip of the sperm tail [6]. In fowl and mammalian spermatozoa, the cytosolic counterpart of Mi-CK is the ubiquitous brain-type BB-CK (cytosolic brain isoenzyme of creatine kinase), which is localized throughout the entire sperm tail [4], as well as in the acrosomal tip [6]. In sea-urchin spermatozoa, however, a unique tail-specific isoenzyme (sea-urchin sperm tail-specific isoenzyme of creatine kinase: TCK) with a molecular mass of 150 kDa has been found, which is the product of a contiguous triplicated CK gene and which is partly associated with the flagellar axoneme [7,8].

Sea-urchin sperm cells are stored in a quiescent state in the gonads, but upon release into seawater, flagellar motility is initiated, and a 50-fold activation of respiration occurs [9,10]. Due to Na^{+}/H^{+} exchange across the sperm plasma membrane, release into seawater is associated with an increase in intracellular pH which, in turn, activates flagellar dynein ATPase. This dynein ATPase utilizes most of the ATP, which in sea-urchin spermatozoa is delivered exclusively by oxidative phosphorylation in a single mitochondrion located in the midpiece of the sperm cell. The transport of high-energy phosphates between mitochondrion and sperm tail has been proposed to be mediated by a 'CK/PCr shuttle' [3,11,12]. The recently solved atomic structure of chicken-heart Mi-CK octamer [13], appears to be consistent with its proposed function for high-energy phosphate channelling from mitochondria to the cytosol [13]. The CK/PCr-shuttle concept suggests that through a close functional coupling of TCK and Mi-CK with dynein ATPase activity and mitochondrial oxidative phosphorylation, respectively, the CK-mediated flux should increase with elevated ATP turnover.

Since sea-urchin sperm cells contain large amounts of CK and can be readily switched, in a co-ordinate fashion, from an inactive to an active state, they represent a relatively simple *in vivo* model to assess the response of CK-mediated flux to changes in free-energy demand. The levels of phosphate metabolites and the intracellular pH can be monitored non-invasively by ^{31}P-NMR spectroscopy. In addition, ^{31}P-NMR magnetization transfer techniques have proven useful in assessing enzyme kinetics in intact cells or tissues, and have often been used to investigate the kinetics of CK-catalysed phosphate exchange [14].

The objective of this study was to assess whether increased ATP turnover, associated with the initiation of motility of intact sea-urchin spermatozoa, affects the steady-state CK-mediated exchange flux, as measured by ^{31}P-NMR saturation transfer.

MATERIALS AND METHODS

Sperm isolation

Sea urchins of the species *Psammechinus miliaris* were purchased from the University Marine Biological Station, Millport, Isle of Cumbrae, Scotland, U.K. Sperm from these sea urchins were isolated by intracoelomic injection of 0.5 M KCl at 12 °C. To obtain inactive spermatozoa, the undiluted, so-called 'dry sperm' (approx. 2.5×10^{10} cells/ml) were diluted 20-fold in 'Na^{+}-free seawater', i.e. a medium containing 450 mM choline chloride, 10 mM KCl, 50 mM $MgCl_2$, 1 mM EGTA, 15 mM Tris and 15 mM Hepes (pH 5.9), and were then centrifuged (6 min, 550 ***g***, 4 °C). The fluffy pellet was resuspended in Na^{+}-free seawater to

Abbreviations used: Cr, creatine; CK, creatine kinase; Mi-CK, mitochondrial isoenzyme of creatine kinase; PCr, phosphocreatine; TCK, sea-urchin sperm tail-specific isoenzyme of creatine kinase.

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a final volume of 10 ml (approx. 1.2×10^{10} cells/ml). To obtain active, motile spermatozoa, dry sperm were washed in 'Ca^{2+}-free seawater', a medium containing 450 mM NaCl, 10 mM KCl, 50 mM $MgCl_2$, 1 mM EGTA, 15 mM Tris and 15 mM Hepes, pH 8.2.

NMR measurements

^{31}P-NMR spectra were recorded at 121.5 MHz on a Bruker MSL300 spectrometer. Measurements on suspensions of intact quiescent sperm cells (approx. 1.2×10^{10} cells/ml) were performed at 10 °C using a 20 mm dedicated ^{31}P probe and a home-built aeration and air-driven stirring device. To maintain aerobic conditions, the suspension was stirred vigorously, while humidified oxygen was bubbled through the upper part of the cell suspension, well above the sensitive area of the NMR radiofrequency coils. Suspensions of active sperm cells were somewhat more dilute (approx. 9×10^{9} cells/ml), to increase the efficiency of aeration.

Spectra were typically obtained by accumulating 200 transients with 90 ° pulses (57 μs) and a relaxation delay of 10 s. A 15 Hz exponential line broadening was applied before Fourier transformation. Steady-state saturation transfer experiments were performed through saturation of the [γ-^{31}P]ATP resonance, using a selective low-power continuous-wave radiofrequency pulse (10 s) before the 90 ° acquisition pulse. Direct spillover of saturation power to neighbouring resonances was generally less than 10 % and was corrected for by recording a control spectrum in which the selective saturation pulse was positioned at the same frequency difference ($|\nu_{PCr} - \nu_{[\gamma\text{-}31P]ATP}|$) downfield of the PCr resonance. The saturation transfer and control spectra were each the sum of 96 scans and were collected in interleaved blocks of eight scans.

The apparent pseudo-first-order unidirectional rate constant for the CK reaction in the direction of ATP synthesis, k_{for}, was calculated from $k_{for} = (1 - M^{+}/M^{0}) \cdot (T_{1,app})^{-1}$, where M^{+} and M^{0} are the integrals of the PCr peak with and without selective saturation of [γ-^{31}P]ATP respectively. $T_{1,app}$ is the apparent longitudinal relaxation time of PCr while saturating [γ-^{31}P]ATP, and was determined by the method of saturation recovery [15]. The intrinsic spin lattice relaxation time ($T_{1,intr}$) of PCr, which would be the T_1 in the absence of any exchange involving PCr, was calculated from: $(T_{1,intr})^{-1} = (T_{1,app})^{-1} - k_{for}$. The apparent longitudinal relaxation time, $T_{1,app}$, for PCr was determined in the presence of selective pre-irradiation of [γ-^{31}P]ATP. Spectra were evaluated by determining the peak areas, using the standard Bruker integration routine of our spectrometer.

Intracellular pH was estimated from the difference in chemical shift between the resonances of intracellular P_i and PCr [16]. Free cytosolic ADP concentrations were calculated on the assumption that CK is near-equilibrium with an apparent K_{eq} of 1.12×10^{9} M^{-1} at 10 °C and at 1 mM free Mg^{2+} concentration [17,18].

Enzyme activities and metabolite concentrations

Spectrophotometric assays were performed on a Beckman DU-65 spectrophotometer either at 25 °C or at 10 °C. CK activity was determined by measuring the rate of ATP synthesis from PCr and ADP (denoted here as the forward CK reaction) at pH 6.9 in a hexokinase/glucose-6-phosphate-dehydrogenase-coupled enzyme assay, as has been described previously [19]. The assay was started by addition of 30 mM PCr and 4 mM MgADP. Total CK activities in whole sperm were determined after permeabilization of sperm membranes with 0.04 % (v/v) Nonidet P40, as has been described previously by Tombes and Shapiro [3].

Sperm cells were extracted by adding ice-cold perchloric acid (final concentration 0.71 M) to a diluted sperm-cell suspension. ATP, PCr and Cr concentrations in neutralized sperm cell extracts were determined spectrophotometrically, by using standard coupled enzyme assays [20,21]. The protein content of each sperm cell suspension was determined before extraction according to Bradford [22]. Absolute metabolite concentrations and cell densities were calculated by assuming a protein content of whole sperm of 1 mg per 6×10^{8} cells and a cytoplasmic volume of 7×10^{-15} litre/cell [12].

Prediction of the CK flux

The exchange flux from PCr to ATP, as detected by NMR, was compared with mathematical predictions of this flux, using the kinetic rate equation for CK, as described by Morrison and Cleland [23]. The following equation was used:

$$\text{Flux} = \frac{V_{max} \cdot [\text{ADP}] \cdot [\text{PCr}]}{D \cdot K_m(\text{ADP}) \cdot K_s(\text{PCr})}$$

$$D = 1 + \frac{[\text{ADP}]}{K_s(\text{ADP})} + \frac{[\text{PCr}]}{K_s(\text{PCr})} + \frac{[\text{ATP}]}{K_s(\text{ATP})} + \frac{[\text{Cr}]}{K_s(\text{Cr})} + \frac{[\text{ADP}] \cdot [\text{PCr}]}{K_m(\text{ADP}) \cdot K_s(\text{PCr})} + \frac{[\text{ATP}] \cdot [\text{Cr}]}{K_m(\text{ATP}) \cdot K_s(\text{Cr})} + \frac{[\text{ADP}] \cdot [\text{Cr}]}{K_m(\text{ADP}) \cdot K_s(\text{Cr})}$$

and where K_s and K_m are the dissociation constants of the binary and ternary substrate–enzyme complexes respectively. Substrate K_m values for CK isoenzymes of *Strongylocentrotus* sea-urchin sperm were taken from ref. [8]. K_s values for these CK isoenzymes are currently unknown. Therefore as an estimate, K_s values from chicken-heart Mi-CK and rabbit muscle cytoplamic CK in solution were used, as reported recently [19].

The kinetic parameters for TCK were as follows: K_m(PCr) = 5.8 mM, K_m(ADP) = 0.13 mM, K_m(ATP) = 0.89 mM, K_s(PCr) = 4.28 mM, K_s(ADP) = 0.14 mM, K_s(ATP) = 2.35 mM and K_s(Cr) = 53 mM. The kinetic parameters used for Mi-CK were: K_m(PCr) = 2.7 mM, K_m(ADP) = 0.055 mM, K_m(ATP) = 0.31 mM, K_s(PCr) = 7.8 mM, K_s(ADP) = 0.06 mM, K_s(ATP) = 1.7 mM and K_s(Cr) = 61 mM.

RESULTS

Typical ^{31}P-NMR spectra of suspensions of inactive and active sea-urchin spermatozoa are shown in Figures 1(A) and 1(C) respectively, and were dominated by the resonances from P_i, PCr and the three phosphate groups from ATP. The spectra demonstrate that the pattern of high-energy phosphate metabolites remained essentially unchanged upon sperm activation. PCr levels and the ratio of PCr to ATP were not significantly lower in activated sperm. PCr/ATP ratios, obtained from the *in vivo* ^{31}P-NMR spectra, were 3.6 ± 0.3 ($n = 11$) in inactive and 3.4 ± 0.5 ($n = 7$) in activated sperm cells (see also Table 1). In accordance with these findings, ^{31}P-NMR spectra showed that sperm activation caused, at most, a slight increase in P_i (Figure 1). These observations are in contrast with those of Christen et al. [2], who observed a rapid and essentially full depletion of PCr with an equivalent increase in P_i upon activation of sperm from the sea-urchin *Strongylocentrotus*. The intracellular pH was calculated from the ^{31}P-NMR spectra and was 6.6 ± 0.1 in quiescent sperm and 7.6 ± 0.1 in activated sperm (Table 1).

Respirometry of dense suspensions of spermatozoa (not shown) demonstrated that the rate of oxygen consumption was very low in Na^{+}-free buffer. In the activation medium, respiratory rates were elevated to maximal levels, since the addition of

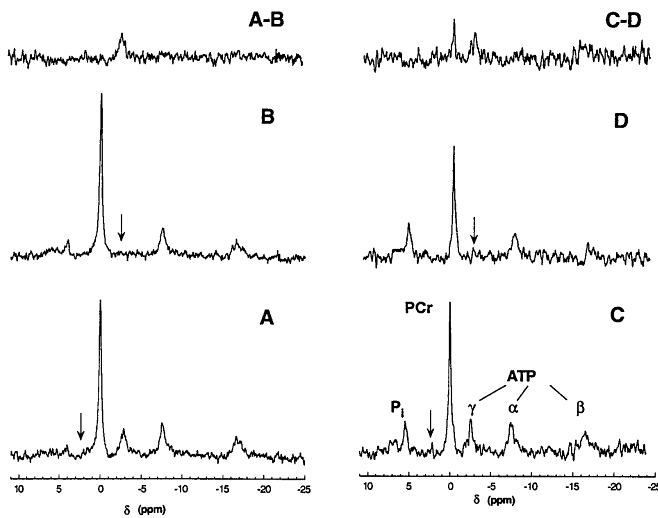

Figure 1 Typical 121.5 MHz ^{31}P-NMR saturation transfer spectra of well-aerated suspensions of spermatozoa from the sea-urchin *Psammechinus miliaris* at 10 °C

Panels (**A**) and (**B**) show inactive spermatozoa suspended in Na^+-free medium, pH 5.8. Panels (**C**) and (**D**) represent active, motile sperm in a buffer containing 450 mM NaCl, pH 8.2. In inactive spermatozoa, selective saturation of [γ-^{31}P]ATP (**B**) caused no detectable saturation of PCr compared with the control (**A**). By contrast, in active spermatozoa, selective irradiation of ATP in (**D**) caused a reduction of the PCr peak, compared with the control (**C**), thus indicating an appreciable PCr–ATP exchange. Panels (**A**–**B**) and (**C**–**D**) show the difference spectra. Arrows denote the frequencies of the low-power saturation pulses.

Table 1 Phosphorus metabolite concentrations and intracellular pH in inactive and active sea-urchin spermatozoa

ATP, Cr and PCr concentrations were determined by standard biochemical assays on perchloric acid extracts (chem). In addition, PCr/ATP and intracellular pH were calculated from ^{31}P-NMR spectra of intact sperm (NMR). Free ADP concentrations were calculated from the CK equilibrium. See the text for further details. Data are means ± S.E.M. (with number of experiments in parentheses).

	Metabolite concentration (mM)	
	Inactive	Active
[ATP] (chem)	7.2 ± 2.1 (4)	7.1 ± 0.8 (7)
[PCr] (chem)	13.2 ± 2.6 (4)	15.1 ± 1.2 (7)
[Creatine] (chem)	18.8 ± 2.6 (4)	22.7 ± 2.4 (7)
[Total Cr] (chem)	32.1 ± 4.5 (4)	37.8 ± 3.0 (7)
Free [ADP]	0.009	0.114
	Ratio	
	Inactive	Active
PCr/ATP (chem)	2.2 ± 0.3 (9)	2.2 ± 0.1 (7)
PCr/ATP (NMR)	3.6 ± 0.3 (11)	3.4 ± 0.5 (7)
	pH	
	Inactive	Active
Intracellular pH (NMR)	6.6 ± 0.1 (11)	7.6 ± 0.1 (7)

mitochondrial uncouplers did not further increase this rate, as has been reported previously [9,10]. Phase-contrast microscopy (not shown) confirmed that sperm cells were immotile in Na^+-free medium at pH 5.8, whereas they were fully motile in the activation medium at pH 8.2.

Independent measurements of the PCr, ATP and Cr concentrations were performed by enzymic assays on perchloric acid extracts of fresh suspensions of inactive and active spermatozoa. As indicated in Table 1, ATP concentrations and PCr/ATP ratios determined enzymically were not significantly different in inactive and active spermatozoa, in agreement with the observations made with NMR. The biochemically determined PCr/ATP ratio was, however, significantly lower than the average PCr/ATP ratio calculated from the NMR spectra (Table 1). This is not unexpected, since ^{31}P-NMR analysis of intact tissue has frequently given higher PCr concentrations and PCr/ATP ratios than chemical analysis of tissue extracts, due to the susceptibility of PCr to hydrolysis during extraction with perchloric acid [24].

The amount of experimental data on absolute PCr and ATP concentrations in sea-urchin sperm is very limited. Tombes et al. [12] have estimated concentrations of 6 mM, 16 mM and 20 mM for ATP, PCr and total Cr respectively for *Strongylocentrotus* sperm. Variations in the levels of these metabolites may be dependent on the species involved, since we found higher average concentrations, as shown in Table 1. In order to estimate intracellular free ADP concentrations, we assumed that CK is near-equilibrium, with an apparent K_{eq} of 1.12×10^9 M^{-1} at pH 7.0 and 10 °C [17,18], that total Cr concentration is 35 mM, and that PCr/ATP ratios are those determined by NMR (Table 1). Thus we estimated that sperm activation is associated with an increase in the free cytosolic ADP concentration, from 9 μM in quiescent to 114 μM in motile sperm (Table 1).

The NMR experiments were optimized to obtain spectra of sufficient signal-to-noise ratio, while keeping the dense cell suspensions well aerated. In this manner, inactive spermatozoa could be maintained in a metabolic steady state for more than 5 h, after which they could still be activated by transfer into Ca^{2+}-free seawater, as corroborated by light microscopy and respirometry. Active sperm cells could be maintained in a steady state for at least 2 h under aerobic conditions. These conditions allowed for steady-state ^{31}P-NMR saturation transfer measurements to assess the kinetics of CK-mediated phosphate exchange in intact, inactive or activated spermatozoa. Figure 1 displays representative ^{31}P-NMR saturation transfer spectra of quiescent (Figure 1B) and motile spermatozoa (Figure 1D). If a significant phosphoryl exchange (of the order of the $T_{1,app}{}^{-1}$ of PCr) between PCr and ATP exists, selective irradiation of the [γ-^{31}P]ATP resonance will result in a decrease in the PCr peak area. Figures 1(A) and 1(B) show that in inactive sperm cells no CK-mediated exchange was detected, whereas in active sperm (Figures 1C and 1D) phosphate exchange was clearly detectable. The apparent pseudo-first-order rate constant for the overall CK reaction in activated spermatozoa was calculated to be 0.13 ± 0.04 s^{-1}. The kinetic and NMR relaxation parameters are summarized in Table 2. The apparent T_1 of PCr was significantly lower in active sperm cells due to the CK-catalysed phosphate exchange. The intrinsic T_1 of PCr was significantly lower in active than in inactive spermatozoa, but the reasons for this discrepancy are unknown. The overall CK flux, although not detectable in quiescent sperm, increased to 3.1 ± 0.5 $mM \cdot s^{-1}$ in fully active sperm at 10 °C, assuming a PCr/ATP ratio of 3.4 and an ATP concentration of 7.1 mM, as listed in Table 1. The exchange rate constant in inactive sperm cells may be below the limit of detection by NMR. Assuming that a 3 % decrease in the PCr peak area during [γ-^{31}P]ATP saturation would be the lower limit

Table 2 Results of ^{31}P-NMR saturation transfer experiments for the forward CK reaction in intact, inactive and active *Psammechinus miliaris* spermatozoa

Measurements were performed at 10 °C and data are means ± S.E.M. (with the number of independent experiments in parentheses).

State	$1-M^{+}/M^{0}$	$T_{1,app}$(PCr) (s)	$T_{1,intr}$(PCr) (s)	k_{for} (s^{-1})
Inactive	0.00 ± 0.01 (8)	2.10 ± 0.10 (4)	2.10 ± 0.10 (4)	< 0.015 (4)
Active	0.16 ± 0.03 (4)	1.25 ± 0.04 (4)	1.49 ± 0.09 (4)	0.13 ± 0.02 (4)

Table 3 NMR-observed CK flux in intact sea-urchin spermatozoa at 10 °C, in relation to the CK flux predicted from the rate equation [23]

The theoretical TCK and Mi-CK exchange fluxes were calculated as described in the Materials and methods section. For 'Predicted CK flux', a total CK V_{max} for inactive sperm of 20 mM · s^{-1}, and for active sperm (intracellular pH = 7.6) of 12 mM · s^{-1}, has been assumed (see the text).

Condition of spermatozoa	Observed CK flux (mM · s^{-1})	Predicted CK flux (mM · s^{-1})	Total CK flux observed/predicted
Inactive	< 0.4 (4)	0.67	< 0.5
Active	3.1 ± 0.5 (4)	3.5	0.9

for NMR detection, we estimated that in inactive spermatozoa k_{for} would be lower than approx. 0.015 s^{-1} (Table 2).

In a separate experiment, sperm cell membranes were permeabilized with 0.04 % (v/v) Nonidet P40, and the specific activity of CK in the spermatozoa was determined enzymically. At 25 °C, the temperature of the standard assay, the specific CK activity was 12.7 ± 3.5 units · mg^{-1} ($n = 15$), which corresponds to previously reported values for sperm of various sea-urchin species [25]. At the temperature of the NMR experiments, i.e. 10 °C, the CK activity was 4.9 ± 1.4 units · mg^{-1} ($n = 15$). Assuming that sperm intracellular water volume is 4.2 μl per mg of protein [12], the CK activity was recalculated to be 20 mM · s^{-1} at 10 °C. TCK and Mi-CK activities in the direction of ATP synthesis are known to decrease at more alkaline pH, above their optimum pH values of 6.9 and 6.7 respectively [8]. In the activated sperm cells (i.e. at pH 7.6) the total forward CK activity is expected to be approx. 40 % lower [8], i.e. approx. 12 mM · s^{-1}.

In order to investigate whether the NMR-detected *in vivo* CK flux might be predicted from the *in vitro* kinetic properties of CK, we calculated CK flux according to the rate equation of Morrison and Cleland [23], as if the enzyme were active in a homogeneous solution. We assumed that *in vitro* kinetic constants for Mi-CK and TCK isoenzymes are similar to reported values for sperm of the *Strongylocentrotus* sea-urchin species [8]. For calculation, PCr/ATP ratios determined by NMR, and ATP concentrations from Table 1 were used. The total Cr concentration was assumed to be 35 mM, which is an average of the chemically determined values in inactive and active sperm cells. Values of binary-complex dissociation constants (K_s) for sea-urchin sperm CK isoenzymes are not available to our knowledge. Therefore, as an estimate, we used previously published values for purified rabbit muscle cytoplasmic CK and chicken heart Mi-CK [19].

The predicted CK flux was 29 % of V_{max} (i.e. 3.5 mM · s^{-1}) in active spermatozoa and 3 % of V_{max} (i.e. 0.67 mM · s^{-1}) in quiescent sperm (Table 3). Variations in intracellular pH and consequently in the free ADP concentration produced the most pronounced effect on the magnitude of the predicted fluxes. The flux/V_{max} ratios that were predicted for sperm tail and mitochondrial CK isoenzymes were almost equal. As it has been reported previously that the specific activities of both CK isoenzymes are equal at pH 7.5 [8], it is expected that Mi-CK and TCK each contribute approx. 50 % to the total CK flux in active sperm.

DISCUSSION

Sea-urchin spermatozoa are instantaneously activated to swim when spawned into seawater. Initiation of motility is mainly a result of activation of dynein ATPase, which is caused by an increase in the intracellular pH of the sperm cells [9,10]. The elevated free cytosolic ADP levels, resulting from increased ATP demand at the flagellar axoneme, may in turn stimulate mitochondrial oxidative phosphorylation, which is the sole ATP-providing pathway in sea-urchin spermatozoa. Here we report that the increased respiratory rates upon sperm activation are accompanied by an elevation of the CK exchange flux.

Intuitively, this observation is in agreement with the original concept of a 'CK/PCr-shuttle' mechanism, in which PCr is a crucial intermediate connecting mitochondrial ATP production and cytosolic ATP utilization [1,26]. Thus far, the most convincing evidence for the existence of a PCr shuttle has been obtained from studying the motility pattern of sea-urchin spermatozoa [3]. Tombes and Shapiro [3] selectively inhibited CK activity with low concentrations of fluorodinitrobenzene and were able to demonstrate that CK function and high-energy phosphate transport via PCr are essential to sustain motility in the distal part of the sperm flagellum. Qualitatively similar results have been obtained with rooster spermatozoa [4]. The transport of high-energy phosphates via a PCr shuttle may be especially important in elongated and functionally polarized cells such as sea-urchin spermatozoa. In these cells, in particular the diffusion of ADP between cytosolic ATPases and mitochondria may be too slow and could potentially limit cellular activity. It is therefore expected that high-energy phosphate transport via PCr and Cr would be more efficient, because of their higher concentrations and diffusivities [27,28]. It is generally accepted that if a PCr shuttle were to be active, CK flux would increase with work load, because of channelling of mitochondrially synthesized ATP to Mi-CK via the ATP/ADP carrier, while in the cytosol ATP from cytosolic CK isoenzymes is channelled towards ATPases [1,26]. The present NMR data, showing increased CK flux with the initiation of sperm motility, therefore seem to corroborate the earlier findings of Tombes and Shapiro [3] that the PCr shuttle is active in sperm.

Comparable NMR studies on mammalian skeletal muscle *in vivo* have shown that the CK flux is apparently not related to work output [29,30]. The observation that variations in ATPase or mitochondrial ATP synthase activity were not accompanied by marked changes in the NMR-observed CK flux has raised some doubt about the importance of PCr-mediated high-energy phosphate transport for muscle energetics, as suggested by the PCr shuttle. It is, however, debatable whether changes in ATPase or mitochondrial ATP synthase activity should be linked obligatorily to adaptations of the CK-reaction velocity. CK flux in cardiac muscle, for instance, exceeds the rate of ATP hydrolysis and synthesis at least 3.5–12-fold [31]. Thus, in this tissue, CK flux generally is not rate limiting to the whole pathway linking ATP production and utilization. Consequently, the CK reaction may be considered to be near equilibrium, which possibly includes any major compartmentalized fractions of CK activity, e.g. at the mitochondrion. However, even if CK flux is independent of workload, a major proportion of high-energy phosphate trans-

port may still proceed via PCr, due to the high CK equilibrium constant [27,28].

In order to investigate how the NMR-measured CK flux in sea-urchin spermatozoa compares with ATP synthesis rates, we examined respirometry data for sea-urchin spermatozoa in the literature. Maximal rates of oxygen consumption in the order of 10 $\mu M \cdot min^{-1}$ per 10^9 sperm, at 10 °C, have been observed [3], which converts into a mitochondrial ATP synthesis rate of approx. 0.14 $mM \cdot s^{-1}$. Since this is almost 25-fold lower than the overall CK flux in active sperm, presumably the CK flux in sea-urchin spermatozoa will be sufficiently high to keep the CK reactants near equilibrium. Under these conditions the calculation of free ADP concentrations from CK equilibrium is valid and we may use free [ADP] to predict CK flux inside the sperm cell. Interestingly, we were then able to predict CK flux in active spermatozoa, as well as the significant increase in CK flux with activation of sea-urchin sperm. Therefore, the increase in CK flux upon sperm activation could well be a kinetic effect brought about by the free [ADP] change and need not necessarily be caused by intimate coupling between CK flux and ATP hydrolysis or oxidative phosphorylation, as would be suggested by the original PCr-shuttle model [26]. The PCr shuttle generally assumes a (temporary) displacement of the CK reaction from thermodynamic equilibrium, in particular at the mitochondrial compartment and at the contractile apparatus, i.e. in the case of spermatozoa the axoneme. It should be stressed that in the latter situation our calculations of free ADP levels and any predictions of the CK exchange flux would become invalid.

Presumably, the CK reaction can only become displaced from equilibrium if the rate of ATP hydrolysis in the sperm flagellum and/or rate of mitochondrial ATP synthesis will be of the same order as the CK reaction velocity. It remains to be resolved whether the NMR saturation transfer experiment, which relies on a steady state, is capable of detecting any imbalance of the CK reaction *in vivo*, when two or more CK isoenzymes are involved. We did not determine the CK reaction rate in the opposite direction (ATP to PCr), since in this direction a quantitative NMR assay is complicated by the involvement of ATP in a number of reactions other than that catalysed by CK. It is to be expected, however, that forward and reverse CK fluxes are equal, given the metabolic steady state that was attained. Also, the NMR experiment is inherently incapable of discriminating between the action of the individual CK isoenzymes. Our calculations predicted that Mi-CK and TCK will contribute equally to the overall CK flux, assuming that specific activities of Mi-CK and TCK are equal at pH 7.5 [8].

The fact that the CK reaction velocity in intact, inactive sea-urchin spermatozoa was not detected by our NMR assay is somewhat surprising. The maximal CK activity measured in permeabilized *Psammechinus* sperm was high, i.e. 20 $mM \cdot s^{-1}$ at 10 °C and pH 6.9. Since we estimated a CK flux of 0.4 $mM \cdot s^{-1}$ (Table 3) as the lower limit of NMR detection, this would imply that CK flux in inactive spermatozoa may be less than 2% of V_{max}. For comparison, in resting mammalian skeletal muscle *in vivo* [29], and in K^+-arrested cardiac muscle *in vitro*, CK flux is readily detected [32]. Like in sperm, CK flux proved not to be detectable by NMR in the resting electric organ of the electric fish *Narcine brasiliensis*, whereas CK was highly active when extracted from the tissue [33]. It has been proposed that inhibitory anions, such as nitrate, nitrite [33], chloride or bicarbonate [30], may reduce the CK flux *in vivo*. Whether such anions may limit CK flux in inactive spermatozoa is still obscure.

Another possibility would be that part of the actual CK flux is not detected by NMR. CK flux detected *in vivo* by ^{31}P-magnetization-transfer techniques has in some cases proven to be significantly lower than values predicted on the basis of the tissue CK activity and Michaelis constants determined in solution [30,32]. It has been proposed that these NMR techniques may not be able to detect the true CK velocity *in vivo* and, in fact, may only observe a particular, yet undefined, fraction of it [34,35]. In sea-urchin sperm cells, substrate turnover may be partially restricted to specific compartments, e.g. due to close functional coupling of part of the flagellar CK activity with dynein ATPase activity at the axoneme, and of Mi-CK activity with mitochondrial oxidative phosphorylation [1].

The rate equation (eqn. 1) for the CK reaction [23], assuming that Mi-CK and TCK obey solution kinetics, predicted an approximately fivefold rise in CK flux, mainly due to the elevation of free [ADP] from 9 to 114 μM. This prediction agrees with our experimental observations. The forward CK flux in active spermatozoa was measured to be 3.1, whereas it was predicted to be 3.5 $mM \cdot s^{-1}$ (Table 3). For the inactive sperm, the calculations suggested that CK flux could in fact be around the detection limit of the NMR measurement. In spite of the fact that uncertainty exists about the validity of some of the kinetic parameters used in the model (eqn. 1), the predictions fit the experimental data surprisingly well and suggest that the increase in free ADP levels upon sperm activation causes the increase in the CK reaction velocity, while the reaction itself remains near equilibrium. It should be noted that the pH dependence of CK activity cannot be an explanation for the observed increase in CK flux, since the forward TCK activity instead decreases by about 45% and Mi-CK activity by 35% between pH 6.6 and pH 7.6 [8].

In conclusion, the significant increase in the rate of CK-mediated phosphate exchange between PCr and ATP upon activation of sea-urchin spermatozoa seems consistent with the existence of a 'CK/PCr shuttle' in these cells [3], constituting an important link for the transfer of high-energy phosphates from mitochondrial oxidative phosphorylation to dynein ATPases along the sperm-tail axoneme. The relative increase in CK flux observed in sea-urchin sperm can, however, also be explained kinetically by a more than tenfold increase in the cytosolic free ADP concentration, assuming that the CK reaction is near equilibrium. Therefore, PCr-shuttle and near-equilibrium concepts for CK function are not mutually exclusive; apparently, the CK reaction in sea-urchin sperm can be near equilibrium, while at the same time CK function is essential for normal sperm motility. The present findings are of general relevance for the interpretation of NMR measurements of CK flux in intact mammalian tissue such as heart and skeletal muscle.

It should be noted that in mammalian sperm only a small or no CK-mediated flux is to be expected, since mammalian sperm have little or no CK at all and thrive mostly on glycolytic breakdown of exogenous seminal plasma substrates [36].

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