Glycolysis plays an important role in energy transfer from the base to the distal end of the flagellum in mouse sperm.

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Since many of studies have been conducted to elucidate the relationship between energy metabolic pathways (glycolysis and respiration) and flagellar motility in mammalian sperm, contribution of glycolysis to sperm motility has not been fully elucidated yet. In the present study, we performed detailed analysis of mouse sperm flagellar motility for further understanding of the contribution of glycolysis to mammalian sperm motility. Mouse sperm maintained vigorous motility by substrates either for glycolysis or for respiration. By contrast, inhibition of glycolysis by alpha-chlorohydrine (ACH) caused significant decrease in bend angle of flagellar bending wave, sliding velocity of outer doublet microtubules and ATP content even in the presence of respiratory substrates (pyruvate or beta-hydroxybutyrate; BHB). The decrease of flagellar bend angle and sliding velocity are prominent in the distal part of the flagellum, indicating that glycolysis inhibition caused the decrease in ATP concentration therein. These results suggest that glycolysis potentially act as a spatial ATP buffering system, transferring energy (ATP) synthesized by respiration at mitochondria located in the basal part of the flagellum to the distal part. In order to validate glycolytic enzymes can transfer high energy phosphoryls, we calculated intraflagellar concentration profiles of adenine nucleotides along the flagellum by computer simulation analysis. The result demonstrated the involvement of glycolysis for maintaining the ATP concentration at the tip of the flagellum. It is likely that glycolysis plays a key role in energy homeostasis in mouse sperm not only through ATP production but also through energy transfer.
**Introduction**

Mammalian sperm flagella require motility for a long period of time from ejaculation to accomplish fertilization (Austin, 1985). For the maintenance of motility during such a long period, mammalian sperm must continue to metabolize extracellular energy substrates for producing ATP. Therefore, elucidation of the correlation between flagellar movement and energy metabolism is very important to understand the functional feature of the mammalian sperm. Furthermore, it has been expected to be applied to the treatment of male infertility and contraceptive technologies.

There are two major metabolic pathways to produce ATP, glycolysis and respiration. Most mammalian sperm must produce ATP to keep vigorous motility by both or one of them, which are localized at different region of sperm. Mitochondria which perform respiration are localized in the mid-piece, the basal limited locus of flagellum. On the other hand, glycolysis works in the principal piece of flagella occupying major part of flagellum, since several glycolytic enzymes have been reported to be localized on the fibrous sheath, a cytoskeletal structure which goes through entire length of the sperm tail (Krisfalusi et al., 2006; Westhoff and Kamp, 1997). Because of higher efficiency of ATP production and an abundance of mitochondria in mammalian sperm, respiration has been considered to be a major source of ATP production.

Recent studies, however, demonstrated that glycolysis plays a major role for ATP production in mouse sperm flagellar movement (Mukai and Okuno, 2004). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of glycolytic enzyme that catalyzes glyceraldehyde-3-phosphate (GAP) to 1, 3-bisphosphoglycerate (1, 3BPG), is
abundantly localized to the fibrous sheath in porcine, human, bovine, equine and murine sperm (Westhoff and Kamp, 1997; Welch et al., 2000). Genetic deletion of sperm specific isoenzyme of GAPDH, GAPDS, using knock-out model gave rise to sperm immotility even in the presence of pyruvate, a respiration substrates, resulting in infertility (Miki et al., 2004). In addition to GAPDS, proteomic studies revealed that multiple glycolytic enzymes (hexokinase, aldolase, phosphoglycerate kinase, enolase, pyruvate kinase and lactate dehydrogenase) were demonstrated to localize in the fibrous sheath in mouse sperm (Krisfalusi et al., 2006). It was also reported that the knock-out mouse of sperm specific glycolytic enzymes, lactate dehydrogenase C (LDHC) and phosphoglycerate kinase 2 (PGK2), resulted in infertility in mouse (Odet et al., 2008; Danshina et al., 2010). These studies supported that glycolysis in the principal piece is a crucial ATP production pathway in mouse sperm.

Another serious problem that must be solved is how ATP synthesized in mitochondria at the base of the flagellum is supplied sufficiently to the distal end of the flagellum, because ATP is necessary at the end of the flagellum for the active bending movement. In sea urchin, the problem is solved by “creatine shuttle”, an energy transporting system from mitochondria at the base to the tip of the flagella (Tombes et al., 1987). On the other hand, such an energy transferring system has not been detected although mouse sperm has a longer flagellum (120 μm) than sea urchin sperm (40 μm) (Kamp et al., 1996). These results suggest that mitochondria-synthesized ATP is not assumed to be supplied to the tip of flagellum sufficiently. Based on these studies, it has been considered that ATP necessary for flagellar movement is produced mainly by glycolysis.

On the other hand, there are some contradicting reports (Ford, 2006; Tanaka et al., 2004).
Tanaka and colleagues (2004) reported that a testis-specific isoenzyme of succinyl CoA transferase (SCOT-t) is expressed in mouse sperm. SCOT-t is necessary to metabolize D-β-hydroxybutyrate (BHB), a substrate of respiration. When BHB was supplemented to the sperm suspension instead of glucose, the percentage of sperm motility was not affected by α-chlorohydrin (ACH), a potent inhibitor of GAPDH. Moreover, it was reported that intracellular ATP concentration was not decreased by ACH in the absence of glucose (Ford and Harrison, 1985; Ford and Harrison, 1986). These results support that respiration is enough to supply ATP for sperm motility.

As mentioned above, there are some contradictory results about the relationship between metabolic pathways and sperm motility. These studies were focused on which metabolic pathway (glycolysis or respiration) was important and dominant for sperm motility, and did not address to the possibilities that these two metabolic pathways may contribute to the flagellar movement differently. To investigate the possibility, more detailed analysis of flagellar movement seems to be necessary.

In the previous studies, flagellar movement was assessed by the percentage of motile sperm or the beat frequency of flagellum. However, these parameters were insufficient to evaluate the “magnitude” of microtubule sliding, an important parameter for evaluating the amount of microtubule sliding. Flagellar bending motion is produced through sliding of the pairs of doublet microtubules by forces produced by dynein arms that hydrolyze ATP. Therefore, microtubule sliding velocity, resulting from the rate of ATP hydrolysis, is directly related to the ATP concentration which is the result of consumption and production. In order to assess the contribution of ATP production pathways to the flagellar movement, sliding velocity
was calculated as a product of beat frequency and bend angle. It was reported that sliding
velocity correlates with ATP concentration (Yano and Miki, 1980; Si and Okuno, 1995).
Therefore, change of sliding velocity could be assumed to reflect the change of ATP
concentrations directly. Furthermore, the local bending along the flagellum is tightly coupled
to the local sliding velocity since the beat frequency of flagellum is constant throughout the
entire length of flagellum (Okuno and Hiramoto, 1976), and thus leads to an evaluation of
local concentrations of ATP therein.

In the present study, correlation between metabolic pathways, especially glycolysis, and
flagellar movement was re-evaluated by detailed motility analysis (measurement of beat
frequency, bend angle, sliding velocity and local bending). Finally, we found that glycolysis
was suggested to function as not only ATP production system, but also energy transferring
system through spatial buffering of ATP.
Results

Change of motility depending on metabolic substrates

First, differences of motility induced by substrates (Glucose, Pyruvate and BHB) were evaluated by detailed analysis of flagellar movement using various parameters (beat frequency, bend angle and sliding velocity of microtubules). Results are summarized in Table 1. Mouse sperm commonly exhibited high motility (10< Hz beat frequency, 30< rad/sec sliding velocity) at 10 mmol l\(^{-1}\) of each substrate, and very little difference of motility parameters (beat frequency, bend angle, percentage of motility, sliding velocity and waveform of flagella) were observed among them.

Effect of glycolysis inhibition on flagellar movement

Sperm maintained vigorous motility for more than 60 min either in the presence of Glucose, pyruvate or BHB (Table 1). This result indicates that mouse sperm can produce sufficient ATP to sustain motility by either glycolysis or respiration alone. Then, we observed the effect of glycolysis inhibition on sperm motility to clarify the contribution of glycolysis on sperm motility. Fig. 1 shows change of beat frequency, bend angle and sliding velocity with time in the presence or absence of α-chlorohydrin (ACH). When glycolysis was inhibited by ACH in the presence of glucose, mouse sperm stopped swimming by 60 minutes (Fig. 1). On the other hand, beat frequency of sperm was not affected by ACH at least for 60 min, when substrate for respiration, BHB or pyruvate, was added to the test solution (Fig. 1A) instead of glucose. However, the bend angle of flagella was significantly inhibited by ACH at 30 and 60 minutes after activation even in the presence of BHB or pyruvate (Fig. 1B). As a result, sliding velocity was significantly reduced by ACH to less than 30 rad/sec at 30 and 60 minutes after
activation (Fig. 1C). Similar results were obtained when glycolysis was inhibited by
2-deoxyglucose (DOG), an analog of glucose that inhibits hexokinase (Hiipakka and
Hammerstedt, 1978; Hyne and Edwards, 1985: Data not shown). Because sliding velocity was
known to correlate with ATP concentration (Yano and Miki, 1980; Si and Okuno, 1995), these
results indicated a decrease in intracellular ATP by ACH even in the presence of respiratory
substrates.

**Effect of glycolysis inhibition on local bending of flagellum**

Glycolysis-inhibited sperm showed unchanged beat frequency and significantly reduced
bend angle in the presence of respiratory substrates. To further characterize the decrease of
bend angle, local bending along the flagellum was investigated in every 10 µm by measuring
the shear angle.

In the presence of respiratory substrates and absence of ACH, local shear angle in the
flagellum increased as the bending wave propagated to the distal end, except for a “singular
point” at 70 µm, where tangent line and reference line synchronously keep rather parallel
(BHB or pyruvate; Fig. 2A, B). On the other hand, sperm whose glycolysis was inhibited by
ACH did not show such an increase along the flagellar axis in the shear angle. The shear angle
of flagellum was almost unchanged from the basal region to the distal end of flagellum (Fig.
2A, B). Similar results were obtained with DOG. These results indicate that inhibition of
glycolysis abolished the increase in the shear angle toward the distal end of flagellum.

Data shown in Figure 2A, B were re-plotted as the ratio of local bending by dividing
shear angle in glycolysis-inhibited sperm by that in the control sperm (Fig. 2C, D). The ratio of
local bending of glycolysis-inhibited sperm was high at the basal region of flagellum (about
0.7), suggesting that the inhibition was low, but was considerably reduced at the tip of
flagellum (about 0.5).

In addition to the evaluation of local bending by the shear angle, local bending was
evaluated by the bend angle in order to eliminate the “singular point” observed in Fig. 2A and
B (Fig. 3). Similar to the result of shear angle (Fig. 2A, B), sperm showed gradual increase in
bending as wave propagated when glycolysis was not inhibited by ACH (filled symbols). By
contrast, glycolysis-inhibited sperm (open symbols) did not show so large increase as those
without ACH, consistent with the results of the shear angles (Fig. 2A and B). These results
indicate that reduction of bending is prominent in the distal region of flagellum.

**Effect of glycolysis inhibition on the content of ANP in sperm**

Glycolysis-inhibited sperm showed decreased bend angles and sliding velocities despite
the presence of respiratory substrates, suggesting a decrease in ATP concentration. In order to
examine whether intracellular ATP was decreased or not, ANP content was directly measured
by reversed-phase high performance liquid chromatography (HPLC) in mouse sperm 30
minutes after activation. Fig. 4 shows the change in the ANP content by the inhibition of
glycolysis.

When glucose was added to the media as a metabolic substrate, a higher content of ATP
(about 0.4 nmol/10^6 sperm), and lower contents of ADP and AMP (approximately 0.16 and
0.07 nmol/10^6 sperm, respectively) were measured than in sperm incubated in the absence of
substrates. Similarly, high contents of ATP and low contents of ADP, AMP were observed in
the presence of respiratory substrates (Pyr and BHB). There was no significant difference
between them. ACH treatment, however, caused a drastic decrease in ATP (p<0.01) content
and increase in AMP content (p<0.01) in the presence of glucose. ACH treatment also caused a
decrease in ATP content and increase in ADP and AMP in the presence of pyruvate and BHB.
These results suggest that inhibition of glycolysis by ACH causes metabolic perturbation even
in the presence of respiratory substrates.

Metabolome analysis

In order to determine the intracellular state of metabolic intermediates, metabolomic
analysis by CE-TOFMS was conducted (table 2). Intracellular ATP content determined by
metabolomic analysis was 0.368 nmol/10^6 sperm. This value was approximately the same as
that determined by HPLC (Fig. 4), indicating the accuracy of metabolomic analysis.
Calculated concentrations of total intracellular ANP and total PGK substrates (total 3PG and 1,
3BPG) were 11.6 mmol l^{-1} and 0.155 mmol l^{-1}, respectively (table 2). The values for the
cytosolic volume used to calculate the intracellular concentration of each parameter was 53.5
fL (Yeung et al., 2002). The values used for following computer simulation were shown in
Table 2.

Computer simulation

As described above, glycolysis inhibition caused the decrease in the sliding velocity at
the distal part of the flagellum, indicating the deficiency of ATP therein in spite of the presence
of respiratory substrates. These results suggest that ATP synthesized by mitochondria at the
base (mid piece) could not supply to the distal part of the flagellum sufficiently when
glycolysis is inhibited. This phenomenon raised the possibility that glycolysis functions as a
spatial buffering of ATP along mouse sperm flagellum, transferring “energy wave” from the
mid piece to the distal end.
In previous studies, it was reported that glycolytic enzymes, particularly phosphoglycerate kinase (PGK), have a potential to transfer energy in muscle cells through buffering of ATP (Dzeja et al., 2004; Dzeja and Terzic, 2003). To assess the possibility that glycolysis has the potential to transport energy to the distal end of the flagellum in mouse sperm, the concentrations of high energy phosphoryls (ATP and ADP) along the flagellum were calculated by simulating the intraflagellar diffusion of high energy phosphoryls in silico. The algorithms of the simulation were based on the simulation model by Tombes et al. that verified energy transfer potential of creatine kinase in sea urchin sperm (Tombes et al., 1987). The diffusing length of flagellum was set to 100 µm although murine sperm has a 120 µm flagellum, because the mid piece, a mitochondria-rich region, is as long as 20 µm. Fig. 5 illustrates the results of calculations of ATP, ADP and ATPase activity profile along the flagellum.

It was reported that adenylate kinase (AK) also participates in energy transfer (Dzeja and Terzic, 2003). Since AK is abundantly present throughout the mouse sperm flagella (Cao et al., 2006), the reaction of AK was also considered in the equation. The parameters of AK used in the simulation were obtained from the data on rabbit muscle AK (Noda, 1973). When the reaction by AK is included and glycolysis (PGK) is excluded in the simulation, ATP at the tip of the flagellum decreased from 11.4 mmol l⁻¹ (basal concentration) to 5.45 mmol l⁻¹, whereas ADP at the tip increased from 0.2 mmol l⁻¹ (basal concentration) to 3.71 mmol l⁻¹ (data not shown). The ratio of the total amount of intraflagellar ATP to the total ADP calculated by computer simulation was approximately 5:2 under this condition where only AK operates in ATP transfer. This ratio was quite different from the ratio of the total intracellular ATP to total
intracellular ADP of glycolysis-inhibited sperm determined by HPLC analysis, which was approximately 5:4 (Fig. 4). When both AK rate and PGK rate were reduced to zero, in other words, when ATP at the distal region was assumed to be supplied only by simple diffusion from the mid piece without ATP buffering, the ratio of the total intraflagellar ATP to the total intraflagellar ADP was calculated to be approximately 5:4, similar to the ratio determined by HPLC in glycolysis-inhibited sperm (Fig. 5, red lines). These results suggest that AK activity may be so low that we can neglect its involvement in the energy transferring system in mouse sperm under physiological conditions despite the presence of AK (Cao et al., 2006). Therefore, in following calculations ANP profiles along the flagellum were done without AK activity (Fig. 5A-C).

When PGK activity was reduced to zero, drastic decrease was observed in ATP concentration from 11.4 mmol l\(^{-1}\) (basal concentration) to 4.32 mmol l\(^{-1}\) and increase in ADP concentration from 0.2 mmol l\(^{-1}\) (basal concentration) to 7.13 mmol l\(^{-1}\) at the tip of flagellum were observed. The resultant activity of dynein ATPase at the tip of the flagellum attenuated from 0.133 mmol l\(^{-1}\)/sec (basal value, this value is constant among calculations) to 0.0738 mmol l\(^{-1}\)/sec. The ATPase activity was calculated from the equation (2) in Materials and Methods. This means that ATPase rate at the tip of the flagellum decreased to approximately 55% of the basal value.

When PGK reaction was included in the equation, a slight increase in ATP concentration from 4.32 mmol l\(^{-1}\) to 4.5 mmol l\(^{-1}\) and a decrease in ADP concentration from 7.13 mmol l\(^{-1}\) to 7.0 mmol l\(^{-1}\) were observed in the presence of 0.155 mmol l\(^{-1}\) PGK substrates (total 3PG and 1,3BPG). The resultant ATPase activity at the tip was 0.0754 mmol l\(^{-1}\)/sec, which is almost the
same to that calculated without the PGK activity. However, the ATPase activity at the tip of the flagellum increased up to 0.0895 mmol l\(^{-1}\)/sec, 0.104 mmol l\(^{-1}\)/sec and 0.131 mmol l\(^{-1}\)/sec when the concentration of the PGK substrates increased to 1.55 mmol l\(^{-1}\), 3.1 mmol l\(^{-1}\) and 6.2 mmol l\(^{-1}\), respectively (Fig. 5C). These results indicate that PGK has a capacity to transfer high energy phosphoryls through spatial buffering of ATP when sufficient glycolytic intermediates are available.

Discussion

Mammalian sperm must metabolize extracellular energy substrates to produce ATP for a long period to accomplish fertilization. Recently, many investigators reported about the relationship between metabolic pathway and flagellar motility in mouse sperm (Mukai and Okuno, 2004; Miki et al., 2004; Ford, 2006; Tanaka et al., 2003), but none of them focused on the different contribution of metabolic pathways to the flagellar movement. In the present study, we performed detailed analysis of mouse sperm flagellar movement using various
parameters to estimate the difference of contribution of metabolic pathways to mouse sperm motility, and revealed that glycolysis has an important role in energy transfer in mouse sperm flagella.

As reported previously (Mukai and Okuno, 2004; Miki et al., 2004; Tanaka et al., 2003), mouse sperm maintained vigorous motility for more than 60 min in the presence of energy substrates (glucose, pyruvate, BHB). There was no difference in motility parameters (beat frequency, bend angle, percentage of motility and sliding velocity) by substrates (Table 1). Since glucose is metabolized by glycolysis alone when sufficient glucose is available (Odet et al., 2011), these results indicate that mouse sperm are able to produce and supply sufficient amount of ATP for maintaining motility by both glycolysis and respiration.

On the other hand, ACH, which inhibits GAPDH through oxidation in cytoplasm (Mohri et al., 1975; Brown-Woodman et al., 1978; Stevenson and Jones, 1985), caused significant decrease in the bend angle and the sliding velocity even in the presence of respiratory substrates such as pyruvate or BHB (Fig. 1). Furthermore, measurements of local bending revealed flagellar bending was severely inhibited by ACH and DOG especially at the distal region of flagellum (Figs 2, 3). These results are inconsistent with the previous study (Tanaka et al., 2004). Detailed analysis of flagellar movement of the present study allowed us to evaluate motility change which had been overlooked in the assessments of the percentage of motile sperm in the previous study.

If the beat frequency is assumed to be constant throughout the flagellum as Okuno and Hiramoto (1976) suggested, a decrease in local bending at a distal part of flagellum indicates a decrease in sliding velocity of doublet microtubule at that locus. Since the sliding velocity
correlates ATP concentration, the decreased bending suggests the decrease in ATP concentration at the distal part of the flagellum in glycolysis-inhibited sperm. By contrast, it is likely that ATP level at basal region of glycolysis-inhibited sperm is unchanged or almost saturated since beat frequency and local bending of basal region was unaffected by ACH. This motility character resembles in sea urchin sperm flagella in which the activity of creatine kinase is inhibited. In this case, creatine phosphate and creatine kinase are indispensable for energy supply from mitochondria located at basal region of flagellum to the distal end (Tombes et al., 1987; Tombes and Shapiro, 1985). Moreover, Shingyoji and colleagues (1995) reported about the relationship among the ATP concentration, beat frequency, bend angle and sliding velocity using a head vibrating technique of demembranated sea urchin sperm as follows; the head of demembranated sea urchin sperm suspended in a certain concentration of ATP were held by suction at the tip of a micropipette and vibrated laterally with respect to head axis. When sperm was vibrated at frequencies higher than undriven beat frequency of flagella, the apparent time-averaged sliding velocity of axonemal microtubules remain constant, with higher frequency being accompanied by decrease in the bend angle (Shingyoji et al., 1995). This phenomenon corresponds with the present study; proximal region of flagella is assumed to contain high concentration of ATP in the presence of respiratory substrates because mitochondria are located at the proximal region of flagella (mid piece), resulting in high sliding velocity therein. Generated bends propagate with a constant beat frequency to the distal end of flagellum. However, decrease in ATP concentration at the distal end of flagellum by glycolysis inhibition causes decrease in sliding velocity at distal end, as a consequence of the decrease in bend angle. Taken together, glycolysis inhibition by ACH (or DOG) probably
induced a decrease in ATP, especially at the distal part of flagellum even in the presence of respiratory substrates.

On the other hand, sperm swimming in the media supplied with respiration substrates without glycolysis inhibition showed local bending and sliding velocity comparable to those observed in sperm supplied with glucose throughout the flagellum. This result suggests sufficient ATP was supplied to the distal end of flagellum. Taken together, it is likely that glycolysis has an important role for the supply of ATP to the distal end of flagellum.

Since the decrease in ATP concentration at distal part of flagellum is assumed to result in the decrease in total ATP in the sperm, total ATP in sperm was measured directly by reversed-phase HPLC. As shown in Fig. 4, the total ATP content was apparently reduced by ACH in pyruvate- and BHB-supplied sperm. By contrast, the ADP and AMP content increased by ACH treatment. Because several mmol l\(^{-1}\) of ADP inhibits dynein ATPase in a competitive manner (Okuno and Brokaw, 1979), the increase in ADP, together with the decrease in ATP concentration, may impair microtubule sliding velocity. Therefore, it is likely that the reduction in sliding velocity by inhibition of glycolysis may be induced by both decrease in ATP and increase in ADP.

It was previously proposed that ATP produced in mitochondria at the base of flagellum is supplied to the distal part of flagellum by simple diffusion (Nevo and Rikmenspoel, 1970). Tombes and colleagues, however, reported that sea urchin sperm could not maintain normal motility and failed in accomplishing fertilization without creatine kinase, an enzyme which catalyzes the reaction that is indispensable for energy-transporting system (Tombes and Shapiro, 1985; Tombes et al., 1987). Since mouse sperm have three times longer flagella (120
µm) than sea urchin sperm (40 µm), it is predicted that ATP produced in mitochondria at the basal region of flagellum cannot be supplied sufficiently by simple diffusion. Nevertheless, creatine kinase was not detected in mouse sperm (Kamp et al., 1996). Furthermore, knock out of ubiquitous mitochondrial creatine kinase in mouse did not impact on sperm motility and fertility (Steeghs et al., 1995). As mouse sperm apparently can supply adequate ATP from base to the tip of the flagellum (Figs 2, 3), another energy supplying system in mouse sperm is predicted.

In skeletal muscle, an energy transferring system by glycolytic enzymes through ATP spatial buffering which is called near equilibrium enzymatic flux network, was proposed recently (Dzeja et al., 2004; Dzeja and Terzic, 2003). In this system, individual glycolytic enzymes work as an ATP spatial buffer, replenishing ATP at the distal part of the flagellum. Sequential buffering reactions by glycolytic enzymes apparently “transfer” a wave of high energy phosphoryls from basal mitochondria to the distal part of the flagellum. Based on their theory, we would like to propose a hypothesis about a new function of glycolysis as energy transferring system in mouse sperm flagella. Schematic model of energy transfer by glycolytic enzymes is illustrated in Fig. 6. In this model, sequential rapid equilibrating reactions catalyzed by phosphoglycerate kinase (PGK) and GAPDH work in concert as a spatial ATP buffer, which transfer “energy wave” from the ATP producing site (mitochondria at mid-piece) to ATP consuming sites (dynein arms along the flagellum) in the flagellum. To realize this ATP spatial buffer, it is essential that enzymes involved in the reaction distribute throughout the flagellum. From this point of view, glycolytic enzymes are suitable for supplying ATP since glycolytic enzymes localize to the fibrous sheath as a complex, which runs along the entire
length of the sperm tail. In addition, it is assumed that reduction in the flagellar bending at the
distal part of the flagellum caused by ACH is attributed to the accumulation of Pi, a product of
ATP hydrolysis, because Pi acts as a competitive inhibitor as demonstrated in demembranated
sea urchin sperm motility although the inhibition is not so strong (Okuno and Brokaw, 1979).

To validate the hypothesis that glycolytic enzyme functions as an ATP spatial buffer,
computer simulation of ANP diffusion along the flagellum was conducted based on the
algorithms by Tombes et al. (1987). In this simulation, we adopted the reaction by PGK only
since reaction of PGK is reported to be particularly important for transfer high energy
phosphoryls (Dzeja and Terzic, 2003). Although AK is abundantly present throughout the
mouse sperm flagella (Cao et al., 2006), we ignore the reaction of AK because of the reasons
described in Results.

The ATPase activity at the distal end attenuated from 0.133 mmol l⁻¹/sec (basal value) to
0.0738 mmol l⁻¹/sec in the absence of PGK reaction. Inclusion of the PGK activity did not
significantly cause the recovery of ATPase activity at the tip of the flagellum (0.0754 mmol
l⁻¹/sec) when the concentration of PGK substrates (total 3PG and 1, 3BPG) were 0.155 mmol
l⁻¹, a value determined by metabolomic analysis (Table 2). Increase in the concentration of
PGK substrates (~3.1 mmol l⁻¹), however, caused a recovery of ATPase activity to 0.104 mmol
l⁻¹/sec. Finally, ATPase activity at the tip did not decrease in the presence of PGK substrates as
high as 6.2 mmol l⁻¹ (0.131 mmol l⁻¹/sec, Fig. 5). These results suggest that a PGK reaction
potentially acts as ATP spatial buffer, transferring energy in mouse sperm flagella when
sufficient PGK substrates are available. In *Lactococcus lactis*, more than 10 mmol l⁻¹ of 3PG
was observed by ¹³C-NMR analysis (Neves et al., 2000). In addition, 3PG and 1, 3BPG
concentrations in mouse brain cells were calculated to be 18.4 and 20.8 mmol l\(^{-1}\), respectively, by computer simulation analysis (Olán et al., 2008). By contrast to these reports, PGK substrates concentration determined by metabolomic analysis in the present experiment was much lower, 0.155 mmol l\(^{-1}\) (Table 2). Since 3PG and 1, 3BPG are highly unstable compounds, it could be speculated that they have been degraded during sample preparation in the present experiment, resulting in underestimation of the concentration of PGK substrates. The actual concentrations of PGK substrates could be enough for energy transfer in mouse sperm.

The calculated ratio of the total intraflagellar ATP to the total ADP in the presence of 1.55 mmol l\(^{-1}\) PGK substrates was 5:2. The ratio of total intracellular ATP to total ADP in glycolysis-non-inhibited sperm determined by HPLC measurements in the present experiments represented 5:2-6:2 (Fig. 4). This ratio, 5:2, is obtained by simulation when PGK concentration is 1.55 mmol l\(^{-1}\). Therefore, the experimental data seems to support that actual value of intracellular PGK substrates are approximately 1.55 mmol l\(^{-1}\).

On the other hand, fitting of the results of computer simulation to the results of flagellar movement analysis suggested a different concentration of PGK substrates. The ATPase activity at the tip of the flagellum calculated from the PGK activity in the presence of 6.2 mmol l\(^{-1}\) PGK substrates was 0.131 mmol l\(^{-1}/s\). By contrast, The ATPase activity at the tip of the flagellum without PGK activity was 0.0738 mmol l\(^{-1}/s\); the ratio between two ATPase values was 0.56. This ratio was similar to the ratio of local bending of glycolysis-inhibited sperm to that of control sperm at the tip of the flagellum, which was approximately 0.5 (see Figs 2, 3). Changes in local bending along the flagellum in the presence of ACH obtained in the present experiments (Fig. 2C and D) and the simulated ATPase activity (Fig. 5C) are superimposed in
Fig. 7. The relatively good coincidence of them suggests that the energy transporting system by means of glycolysis could be employed in the flagellum. These results suggest that realistic intracellular concentration of PGK substrates is approximately 6.2 mmol l⁻¹. Taken together, the concentration of PGK substrates were assumed to be 1.5-6.2 mmol l⁻¹. Under present experimental conditions, such concentrations of PGK substrates might realized by high concentrations of respiratory substrates which would stop glycolytic flux, or alternatively high concentrations of respiratory substrates would replenish PGK substrates by reverse reaction that are conventionally considered irreversible under physiological condition. Although the results from computer simulation analysis strongly suggest that PGK has a potential to functions as ATP spatial buffer, transferring energy in mouse sperm flagella, further studies to determine the accurate value of PGK substrates concentration are necessary.

In conclusion, it was suggested that the ATP content in distal part of flagellum is reduced by glycolysis inhibition even in the presence of substrates of respiration. Based on this result, we proposed a new energy transfer system based on spatial buffering of ATP by glycolytic enzymes in mouse sperm. Further investigations about this new function of glycolysis in mouse sperm are needed, and would shed a light on energy homeostasis not only in mammalian sperm physiology, but also in diverse motile cilia and flagella.
Materials and Methods

Sperm preparation

Sperm were obtained from the cauda epididymis of 8-15 wk old ICR male mice (Mus Musculus) in accordance with the guidance of the University of Tokyo. The cauda epididymis was excised and punched with needles. Epididymal sperm was gently squeezed out and diluted into 100 µl of sucrose solution (300 mmol l⁻¹ sucrose, 10 mmol l⁻¹ Hepes-NaOH, pH 7.4), in which sperm exhibited very low activity (about 1 Hz of beat frequency), referred to as the initiated sperm by Fujinoki et al (2001). The sperm suspension was diluted with the test solution for the following experiments. The test solution contains 150 mmol l⁻¹ NaCl, 5.5 mmol l⁻¹ KCl, 0.4 mmol l⁻¹ MgSO₄, 1 mmol l⁻¹ CaCl₂, 10 mmol l⁻¹ NaHCO₃, 10 mmol l⁻¹ Hepes-NaOH (pH 7.4), and 10 mmol l⁻¹ of metabolic substrates, such as glucose (Glc), pyruvate (Pyr), and BHB. One volume of sperm suspension was diluted into 20 volume of each test solution and was incubated in CO₂ incubator (37°C, 5% CO₂) for observation.

For the inhibition of glycolysis, either 10 mmol l⁻¹ of ACH or 10 mmol l⁻¹ of DOG was added to each test solution.

Analysis of sperm motility

After the incubation in the test solution containing metabolic substrates and inhibitors, an aliquot of sperm suspension was placed onto a prewarmed glass slide at 37°C and covered with a coverslip for observation with a microscope.

For the analysis of microtubule sliding velocity, sperm with their head attach to the glass surface was observed using a phase-contrast microscope (Diaphoto, Nikon, Tokyo, Japan), captured by a CCD camera (CR-20, Video Device, Chiba, Japan) and recorded by a video
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To analyze the sliding velocity, the flagellar waveforms were carefully traced by hand from a video monitor onto a transparent plastic film. The beat frequency was calculated from the number of video fields required to complete one beat cycle determined by using the traced waveform. The bend angle of a flagellum was determined by measuring the angle between the tangents at two adjacent points of inflection when flagellar bends reach at the center of flagellum (Fig. 8A). Bend angle was measured for both principal bends (Fig. 8A-a) and reverse bends (Fig. 8A-b), and data were expressed as a sum of both values. Microtubule sliding velocity was defined as the multiplication of beat frequency and bend angle.

For the measurement of the shear angle, sperm with their head attached to the glass surface was recorded digitally using a phase-contrast microscope and a computer-driven high-speed camera (HAS-220CH, DITECT, Tokyo, Japan). The shear angle of sperm flagella was analyzed by “Bohboh”, a flagellar movement auto-analyzing software kindly provided by Dr. Shoji Baba (emeritus professor at Ochanomizu University, Tokyo, Japan). Shear angle was defined as the angle between the reference line at the base of the flagellum, usually parallel to the head axis, and the tangent at a point along the flagellum (See Fig. 8B). In the present experiment, shear angle was determined every 10 µm on the flagellum from the base to the tip for 3 cycle of beating. Then, difference between the maximum and minimum values was defined as a local amount of microtubule sliding per one beat cycle (Fig. 8B).

A local bend angle, defined as the angle between the tangents at the two inflection points around each vertex of the waveform, was determined on the images captured digitally by high-speed camera. The analysis was conducted manually as the determination of bend angle.
The distance of bending vertex from the base of flagellum was determined digitally by Bohboh software.

**Measurement of ATP content by reversed phase HPLC**

Evaluation of ATP content in mouse sperm cells was performed by reversed-phase HPLC (LC10VP series, Shimadzu, Kyoto, Japan) with slight modifications (Mukai and Okuno, 2004; Samizo et al., 2001). Sperm suspended with each test solution in microtubes were incubated in a CO₂ incubator (5% CO₂, 37°C) for 30 min. One-tenth volume of 3% ice chilled perchloric acid (PCA) was added to each sperm suspension to remove proteins, and the microtubes were placed on ice for 10 min. After centrifugation (10000 x g, 10 min, 4°C), the supernatant was filtered with a membrane of 0.22 µm pore size. Filtered solution was neutralized with phosphate buffer, and 25 µl of neutralized solution was applied to a reversed-phase HPLC column (Phenomenex Luna 5 µC18, 4.6 × 150 mm; Shimadzu GLC, Tokyo, Japan). The mobile phase contained 20 mmol l⁻¹ potassium phosphate (pH 6.8), and 5 mmol l⁻¹ of tetrabutyl-ammonium hydroxide, and 20% methanol. The number of sperm cells was counted in each sample, and the content of ATP was represented as nmol per 10⁶ sperm.

**Metabolomic analysis**

To determine the content of glycolytic intermediates, metabolome analysis was performed by a capillary electrophoresis electrospray ionization time-of-flight mass spectrometry (CE-TOFMS) method. One ml of sperm suspension in sucrose solution was diluted into 10-fold volumes of each test solution containing 1 mmol l⁻¹ glucose or 10 mmol l⁻¹ pyruvate with or without 10 mmol l⁻¹ ACH, and incubated for 30 minutes at 37°C. After incubation, 75% percoll was added to the bottom of the tube and centrifuged for 5 minutes at 900 x g, 4°C.
Sperm were observed in the layer between the test solution and percoll. Then the supernatant test solution was discarded and the resultant was washed with 5% (w/w) mannitol solution to remove electrolytes. After centrifugation (900 × g, 4°C, 2.5 minutes), supernatant was discarded and 1 ml of ice-cold methanol was added to fix sperm. Methanol-fixed sperm samples were separated by capillary electrophoresis and the amount of each intermediates are quantified by Mass spectrometry. CE-TOFMS analysis was performed by Human Metabolome Technologies Inc. (Yamagata, Japan).

**Computer simulation analysis of diffusion of ATP and high energy phosphoryls**

Calculation of high energy phosphoryls diffusion along the flagellum is performed based on the algorithms previously reported by Tombes et al (1987). In the model, each molecular species diffusing along the flagellum is governed by a diffusion equation,

\[
\frac{\partial C_{(s,t)}}{\partial t} = D \frac{\partial^2 C_{(s,t)}}{\partial s^2} + Q(s,t) \tag{1}
\]

where \(C_{(s,t)}\) represents the concentration of the diffusing species at position \(s\) and time \(t\). \(D\) is the relevant diffusion coefficient, and \(Q\) is the rate of production of the species by chemical reactions. \(Q\) consisted of three reactions, specified by \(Q_1, Q_2\) and \(Q_3\), are described below.

\(Q_1\) is the dynein ATPase rate. Since the use of ATP by flagella is tightly coupled to motility, the relationship between the ATPase rate and the ATP concentration is assumed to be similar to the relationship between flagellar beat frequency and ATP concentration.

\[
Q_1 = Q_{1F}/\left(1 + K_1\left(1 + \frac{[ADP]}{K_i}/[ATP]\right)\right) \tag{2}
\]

where \(K_1\) is the ATP concentration for half-maximal beat frequency, and \(K_i\) is the constant for competitive inhibition of ATPase rate (beat frequency) by ADP. The maximum rate, \(Q_{1F}\), obtained as value of 0.134 mmol l⁻¹/sec, based on measurements of glucose.
consumption by mouse sperm with the assumption that glucose is metabolized only by
glycolysis (Odet et al., 2011). K1 value of 0.14 mmol l\(^{-1}\), obtained from the measurements on
demembranated ram sperm, was kindly provided by Dr. Sumio Ishijima (Tokyo Institute of
Technology). A value of 0.28 mmol l\(^{-1}\) was used for Ki, based on the measurements indicating
that the value of Ki for demembranated sea urchin sperm flagella was twice the value of K1
(Okuno and Brokaw, 1979). The reverse reaction for the ATPase is neglected.

\[ Q_2 = \frac{\text{rate for the glycolytic reactions}}{\text{Because enzyme that catalyze the first step of}} \]

ATP producing reaction (PGK) is reported to be particularly important for transferring high
energy phosphoryls (Dzeja et al., 2004), only the reaction by PGK is included. This may be a
good assumption, as glycolytic enzymes exist in a complex manner in spermatozoa (Westhoff
and Kamp, 1997). The reaction by PGK is as follows:

\[ \text{ATP} + 3\text{PG} \rightarrow \text{ADP} + 1,3\text{BPG} \]  

where 3PG represents 3-phosphoglycerate and 1, 3BPG represents 1, 3-bisphosphoglycerate.

For this reaction, both forward and reverse rates are considered:

\[
Q_2 = \left( \frac{Q_{2F}^{3\text{PG}[\text{ATP}]}}{K_{mT}K_{mD}} - \frac{Q_{2R}^{1,3\text{BPG}[\text{ADP}]}}{K_{mT}K_{mD}} \right) + \frac{1 + \frac{[\text{ATP}]}{K_{mT}K_{mD}} + \frac{[1,3\text{BPG}][\text{ADP}]}{K_{mT}K_{mD}} + \frac{[\text{ATP}]}{K_{mT}K_{mD}} + \frac{[3\text{PG}]}{K_{mT}K_{mD}} + \frac{[1,3\text{BPG}]}{K_{mD}} + \frac{[\text{ADP}]}{K_{mD}}}{1 + \frac{[3\text{PG}][\text{ATP}]}{K_{mT}K_{mD}} + \frac{[1,3\text{BPG}][\text{ADP}]}{K_{mT}K_{mD}} + \frac{[\text{ATP}]}{K_{mT}K_{mD}} + \frac{[3\text{PG}]}{K_{mT}K_{mD}} + \frac{[1,3\text{BPG}]}{K_{mD}} + \frac{[\text{ADP}]}{K_{mD}}} \right)
\]

Values used for the parameters in this equation are given in Table 2.

\[ Q_3 \text{ is the rate for the adenylate kinase (Noda, 1973).} \]

\[ \text{ATP} + \text{AMP} \rightarrow 2\text{ADP} \]  

For this reaction also, both forward and reverse rates are considered.
The parameters used to calculate in this equation ($Q_{3F}$, $Q_{3R}$, $K_N$, $K_M$, $K_A$), which are originated from rabbit muscle fiber, are given in Table 2.

For each species, $Q$ is the sum of the relevant rates as follows: For ATP: $Q = -Q_1 - Q_2 - Q_3$, for ADP: $Q = Q_1 + Q_2 + 2Q_3$, for AMP: $Q = -Q_3$, for 3PG: $Q = -Q_2$, for 1, 3BPG: $Q = Q_2$.

If the diffusion coefficients for ATP, ADP, and AMP and those for 3PG and 1, 3BPG are assumed to be almost equivalent, there will be no gradient of total adenine nucleotide or total 3PG concentration along the flagellum. Thus, AMP can be obtained from (total adenine nucleotide – ATP – ADP) and 3PG can be obtained from (3PG + 1, 3BPG) – 1, 3BPG.

To solve the system of partial differential equations, three equations were integrated forward with time until a steady equilibrium solution was obtained. Concentrations at the basal end of the flagellum were held constant, and no fluxes were allowed past the distal end of the flagellum. Then, for each species, equation (1) was converted to

$$C_{(s,t+\Delta t)} = C_{(s,t)} + D \left[ \frac{C_{(s-\Delta s,t)} - 2C_{(s,t)} + C_{(s+\Delta s,t)}}{\Delta s^2} \right] \Delta t + Q_{(s,t)} \bullet \Delta t \cdots \cdots \cdots \cdots \cdots \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ (7)$$

The length interval, $\Delta s$, was 1 µm in all the results shown, and the time interval, $\Delta t$, was normally 0.5 msec. The simulation program was written and performed by computer software Mathematica (Wolfram Research, Champaign, IL, USA).

**Statistical analysis**
Values for sliding velocity, shear angle, and ATP content were expressed as the mean and standard error of the measure (s.e.m.). Statistical tests were performed using Student’s t-test for testing differences of content of adenine nucleotides. In the other experiments, data were analyzed by ANOVA and post-hoc Tukey-Kramer test.

**Reagents**

α-chlorohydrin was purchased from Sigma-Aldrich (St. Louis, U.S.A.), β-hydroxybutyrate was from MP Biomedicals (California, U.S.A.), percoll was from GE Healthcare (Chalfont St Giles, UK.), and the other chemicals were purchased form Wako Pure Chemicals Co. ltd. (Osaka, Japan).

**Acknowledgements**

We are grateful to Dr. Shoji A. Baba of Ochanomizu University for providing flagellar movement auto-analyzing software “Bohboh”. We thank Dr. Sumio Ishijima of Tokyo Institute of Technology for providing us K1 value of demembranated ram sperm.

**Funding**

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**Abbreviations**

ACH: α-chlorohydrin
ADP: adenosine diphosphate
AMP: adenosine monophosphate
ATP: adenosine triphosphate
BHB: β-hydroxy butyrate
1, 3-BPG: 1, 3-bisphosphoglycerate
DOG: 2-deoxy-D-glucose
GAP: glyceraldehyde-3-phosphate
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GAPDS: glyceraldehyde-3-phosphate dehydrogenase, spermatogenic
Glc: glucose
3PG: 3-phosphoglycerate
PGK: phosphoglycerate kinase
Pi: inorganic phosphate
Figure legends

Figure 1: Effect of ACH on sperm flagellar motility in the presence of various substrates.

Sperm was diluted into the media containing substrates only or substrates and inhibitor. Then, change in beat frequency (A), bend angle (B) and shear angle (C) with time-course was plotted. Both the shear angle and the bend angle are represented as the averaged absolute values obtained from both the principal and reverse bend. Beat frequency was not significantly reduced by ACH when pyruvate or BHB was present in the media. By contrast, bend angles and shear angles were significantly reduced by ACH even when pyruvate or BHB was present in the media. Concentrations of substrates and inhibitor were 10 mM. Vertical bars represent SEM. N>9 Asterisks indicate significant difference from control sperm (*: p<0.05, **: p<0.01). Glc: glucose, Pyr: pyruvate. ACH: α-chlorohydrin

Figure 2: Changes of local bending along the glycolysis-inhibited sperm flagella

Sperm were diluted into media containing respiration substrates (A: BHB; B: Pyr) and glycolysis inhibitor (ACH or DOG), then allowed to swim in the media for 1 hour in a CO₂ incubator (37°C, 5% CO₂, 95% air). After incubation, local shear angle was measured with computer software Bohboh. Changes of shear angle are plotted at indicated distance from the head (A, B). Data shown in (A, B) were expressed as the ratio of the shear angle in control to that in the glycolysis-inhibited sperm (C, D). Bending decreases in the distal part of flagellum. The concentration of each substrate and inhibitor was 10 mM. Bars represent SEM. N=5. Asterisks indicate significant difference from control sperm (*: p<0.05, **: p<0.01).
Sperm diluted into media containing respiration substrates and glycolysis inhibitor (ACH), then allowed to swim in the media for 1 hour in a CO₂ incubator (37°C, 5%CO₂, 95% air). After incubation, local bend angle was determined for flagella of sperm attached to the glass surface by the head. Measurement was performed as described in Materials and methods. Data are shown as scattergram. ACH-treated sperm (open characters) showed reduced bend angles compared with non-treated controls (filled characters). The concentrations of each substrate and inhibitor were 10 mM.

Glc: glucose, Pyr: pyruvate. ACH: α-chlorohydrin

Content of ANPs are measured by Reversed-phase HPLC 30 minutes after activation. Decrease in the ATP content and increase in the ADP and AMP contents by ACH were observed even in the presence of respiratory substrates. The concentration of each substrate and inhibitor was 10 mM. Data are means of 15 mice ±SEM. Asterisks indicate significant difference from control sperm (*: p<0.05, **: p<0.01).

Glc: glucose, Pyr: pyruvate. ACH: α-chlorohydrin

Calculated concentration profiles of ATP (A) and ADP (B), and calculated profiles of ATPase activity (C) without adenylate kinase activity using the parameters given in Table 2 and equation indicated by Tombes et al. (1987) are shown.
Figure 6: Schematic model of energy transfer system by glycolysis

Schematic models of energy transporting system in sperm flagella by glycolysis is illustrated by modifying energy transfer system proposed by Dzeja and Terzic (2003) to sperm flagella.

PGK: phosphoglycerate kinase
GAPDS: glyceraldehyde-3-phosphate dehydrogenase, spermatogenic
GAP: glyceraldehyde-3-phosphate
3PG: 3-phosphoglycerate
1, 3-BPG: 1, 3-bisphosphoglycerate
NAD: nicotinamide adenine dinucleotide (oxidized)
NADH: nicotinamide adenine dinucleotide (reduced)
Pi: inorganic phosphate

Figure 7: Superimposed schematic model of energy transporting system without glycolysis to the flagellar local bending inhibited by ACH

Simulated ATPase activity along flagellar axis in the absence of energy transporting system by glycolysis (Fig. 6C) is superimposed to the relative change in flagellar local bending when glycolysis is inhibited by ACH. Results from BHB + ACH (Fig. 3 C) and Pyruvate + ACH (Fig. 3 D) are averaged.

Figure 8: Methods for analysis of flagellar movement.

(A) Analysis of bend angle. The bend angle of a flagellum was determined as follows: First,
measuring the angle between the tangents at two adjacent inflection points when flagellar bends reach at the center of flagellum (white circle). The angle was measured for both principal bends (a) and reverse bends (b), and bend angle is defined as a sum of both values. The bend angle was measured three times individually, and the bend angle of each spermatozoon was determined as a mean of them.

(B) Analysis of shear angle. Shear angle at point c in left picture was defined as the angle between the reference line at the base of the flagellum, usually parallel to the head axis, and the tangent at a point c. In the present experiment, shear angle was determined every 10 µm on the flagellum from the base to the tip for 3 cycle of beating (plotted in right graph). Then, difference between the maximum and minimum values (arrows in right graph) was defined as a local amount of microtubule sliding.
References


Table 1. Difference of beat frequency, bend angle, sliding velocity and waveform of sperm flagella depends on energy substrates.

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<tr>
<td>Bend Angle (rad)</td>
<td>2.55±0.12</td>
<td>2.62±0.07</td>
<td>2.63±0.16</td>
</tr>
<tr>
<td>Sliding Velocity (rad/sec)</td>
<td>41.0±1.91</td>
<td>38.1±1.60</td>
<td>42.6±2.26</td>
</tr>
<tr>
<td>Motility (%)</td>
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<td>58.8±2.43</td>
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</tr>
<tr>
<td>Waveform</td>
<td>![Waveform Image]</td>
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Table 2. Parameters used for computations of Pi transport in flagella

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<td>Flagellar length</td>
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</tr>
<tr>
<td>Diffusion coefficient for ANP</td>
<td>60 µm²s⁻¹</td>
<td>Takao and Kamimura (2008)</td>
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<td>104 µm²s⁻¹</td>
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<td>PGK substrates concentration (Total 3PG and 1, 3BPG)</td>
<td>0.155 mmol l⁻¹</td>
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<td>Kᵣ₊ᵣ (PGK2 Kᵣ value for 1, 3BPG)</td>
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Figure 4: Effect of glycolytic inhibitor on the content of ANP in sperm.
(A) ATP concentration profile along flagellum

(B) ADP concentration profile along flagellum
without PKG activity

- 0.155 mmol l-1 PGK substrates
- 0.775 mmol l-1 PGK substrates
- 1.55 mmol l-1 PGK substrates
- 3.1 mmol l-1 PGK substrates
- 4.65 mmol l-1 PGK substrates
- 6.2 mmol l-1 PGK substrates
(C) ATPase activity profile along flagellum

Figure 5: Simulation of high energy phosphoryls diffusion along the flagellum.
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Difference in beat frequency, bend angle, sliding velocity, percentage of motile sperm and typical waveform by substrates are indicated. Data are represented as mean value ± s. e. m. No significant difference in motility parameters was recognized. Concentration of substrates was 10 mmol l⁻¹.
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