Inhibition of the ATP-induced reactivation of demembranated hamster spermatozoa by the action of free ATP^{4-} and MgATP^{2-}

C. H. Yeung

*Max Planck Clinical Research Unit for Reproductive Medicine, Steinfurterstrasse 107,
D-4400 Muenster, Federal Republic of Germany*

Summary. Hamster spermatozoa collected from the caput and cauda epididymidis were washed, diluted in a medium containing Triton X-100 to dissolve the cell membrane and reactivated with various concentrations of MgSO_4 and ATP. Stepwise increase in the concentrations of free ATP^{4-} from 0.08 to 1.1 mM at constant concentrations of MgATP^{2-} caused a dose-dependent delay of reactivation but the maximal percentage of motile spermatozoa was inhibited only at 1.1 mM. The inhibitory effect on caput spermatozoa was greater than that on cauda spermatozoa. When concentrations of ATP^{4-} were fixed at 0.2 mM, 2.9 mM- MgATP^{2-} suppressed the reactivation of cauda spermatozoa. When compared to 0.9 mM- MgATP^{2-} , reactivation of caput spermatozoa was delayed at 1.9 mM and almost completely blocked by 2.9 mM- MgATP^{2-} . Inhibition of cauda sperm reactivation by ATP^{4-} and MgATP^{2-} were both prevented by the presence of trypsin (50 ng/ml). Incubation of cauda spermatozoa in the reactivation medium for 1 and 2 min before the addition of ATP progressively reduced the inhibitory effect of ATP^{4-} ; inhibition by MgATP^{2-} was reduced to a lesser extent. Addition of 100 μM -cyclic AMP to the medium abolished the delay of reactivation by ATP^{4-} but not that by MgATP^{2-} . Before reactivation occurred, inhibitory concentrations of ATP^{4-} and MgATP^{2-} both induced large-angle coiling of sperm tails but in opposite direction to each other with reference to the asymmetric sperm head. The results suggest that free- ATP^{4-} and superoptimal concentrations of MgATP^{2-} inhibit flagellar movement by different mechanisms.

Introduction

Flagellar movement of spermatozoa is based on the active sliding of microtubules (see reviews by Satir, 1979; Brockaw, 1982) as a result of mechanochemical coupling of ATP hydrolysis by dynein ATPase (see review by Warner & Mitchell, 1980) which is located periodically along the outer doublets of the 9 + 2 axial filament structure of the sperm tail. Using the technique of extracting the membrane from spermatozoa with Triton X-100 and reactivating them with Mg^{2+} and ATP (Gibbons & Gibbons, 1972), Yeung (1986) has demonstrated temporary inhibition of the initiation of reactivated motility of hamster spermatozoa by increasing the concentration of ATP from 1 to 3 mM. This confirmed the observations of suboptimal reactivation of other mammalian spermatozoa when concentrations of ATP exceed the optimum (dog: Tash & Means, 1982; ram: White & Volgmayr, 1986) and may explain the discrepancies in the literature concerning the correlation of sperm motility with the intracellular concentrations of ATP (Rogers & Morton, 1973; Foulkes & MacDonald, 1979; Suter *et al.*, 1979; Levin *et al.*, 1981; Ke & Tso, 1982; Calamera *et al.*, 1982). In the present study the inhibitory action of ATP was further investigated.

Materials and Methods

Sperm preparation. Mature male golden hamsters (*Mesocricetus auratus*, 90–130 g body weight, kept in 12 h light: 12 h dark with water and food-pellet intake *ad libitum*) were anaesthetized with urethane (1.8 g/kg). Each epididymis was dissected in culture Medium 199 (Gibco, Paisley, U.K.); either the whole decapsulated caput region or a length (about 5 cm) of untraveller distal cauda tubule was removed. Spermatozoa were released into Ca-free phosphate-buffered solution (pH 7.2) from coarsely minced caput tubule and from the cauda by gently squeezing 1-cm tubule segments. The spermatozoa were washed through 5% Ficoll (M_w 400 000) in the phosphate buffer at $500 g_{max}$ for 5 min, resuspended in the buffer with sperm concentration adjusted to about 3×10^7 /ml and kept on ice. Washed spermatozoa were used within 70 min of resuspension. Each suspension provided material for about 5 different treatments which were randomized with respect to their order of application in different experiments. All chemicals used were from Sigma Chemie (Munich, FRG) unless stated otherwise.

Demembration and reactivation. A 3- μ l sample of washed sperm suspension was diluted in 0.5 ml of the demembration and reactivation medium (Solution R, containing 230 mM-sucrose, 15 mM-potassium glutamate, 0.5 mM-EDTA, 1 mg polyvinylpyrrolidone (M_w 40 000)/ml, 0.04% Triton X-100, 20 mM-Tris-HCl adjusted to pH 7.9) with various amounts of $MgSO_4$. In some experiments, cyclic AMP (100 μ M), trypsin (type III-S; 50 ng/ml) or soybean trypsin inhibitor (type I-S; 10 μ g/ml) was included in Solution R. Adenosine-5'-triphosphate (ATP; disodium salt, vanadium-free; Sigma Chemie or Boehringer Mannheim, Mannheim, FRG) was added after 1 or 2 min, or was already present in the medium when no preincubation time is indicated. The total concentrations of ATP and Mg needed to give the desired concentrations of free ATP^{4-} and $MgATP^{2-}$ were calculated (Bartfai, 1979) using the association constants (log k) of 4.04, 6.32 and 1.9 for $MgATP^{2-}$, $MgEDTA^{2-}$ and magnesium glutamate respectively (see reactivation conditions in Table 1).

Table 1. Total concentrations (mM) of ATP (disodium salt) (ATP_t) and $MgSO_4$ (Mg_t) added to Solution R to test the effects of increasing concentrations of free ATP^{4-} (A–D) and $MgATP^{2-}$ (E–G) on the reactivation of demembrated hamster spermatozoa

Reactivation condition	ATP^{4-}	$MgATP^{2-}$	Mg^{2+}	ATP_t	Mg_t
A	0.08	0.92	1.08	1.00	4.00
B	0.36	0.64	0.16	1.00	1.50
C	0.71	0.79	0.10	1.50	1.50
D	1.11	0.89	0.07	2.00	1.50
E	0.19	0.91	0.43	1.10	2.41
F	0.21	1.89	0.82	2.10	4.32
G	0.22	2.88	1.20	3.10	6.24
H*	0.08	1.02	1.15	1.10	4.31
I*	0.05	1.05	1.88	1.10	6.26

*Controls for Mg^{2+} and Mg_t as used in condition G.

Measurements of motility. An aliquant of each reactivated sperm preparation was placed in an 80 μ m deep siliconized slide chamber (prepared as described by Yeung, 1984) and examined at room temperature (21–25°C) using a Zeiss microscope with a $\times 10$ objective, condenser setting phase 3 and pseudo-dark-field optical conditions. Video-recording of each sample, in connection with a digital timer (time set at zero when spermatozoa were first exposed to exogenous ATP), was made by scanning across the chamber at random starting 1 min after the exposure of spermatozoa to ATP until the percentage of motile cells had apparently reached its peak and started to decline, or for 11 min when it was difficult to judge during this period of recording whether percentage motility had reached the maximum. Spermatozoa with oscillating flagella were counted as motile and percentage motility in each minute of recording was measured by counting all the cells (80–120) scanned. For each reactivation preparation, the time required to reach 75% of the maximal percentage motility was taken to reflect the overall rate of initiation of motility.

Statistical analysis. Statistical evaluation of the results was performed by analysis of variance and comparisons between treatment groups by partition of treatment sum-of-squares (Parker, 1979). Time intervals measured in integral minutes were transformed into their square-root values before analysis. Wilcoxon's Stratified test was used in the comparison of the sensitivity to the inhibitory effects of increasing concentrations of ATP between caput and cauda spermatozoa.

Results

Effect of free ATP^{4-}

The majority of spermatozoa collected from the distal cauda and diluted into Medium 199 were motile ($68.4 \pm 4.5\%$, mean $\pm$ s.d., $n = 5$). They were immediately immobilized in the demembration solution but could resume regular flagellation in the presence of ATP depending on the reactivation conditions. In the first group of experiments, when the calculated concentrations of free ATP^{4-} present in the reactivation medium increased from 0.08 to 1.11 mM, although those of $MgATP^{2-}$ remained relatively constant at 0.64–0.92 mM (conditions A–D in Table 1), the rate of reactivation of the sperm preparation as a whole was decreased (Fig. 1). At the lowest concentration of ATP^{4-} , over 75% maximal reactivation was always detected after 1 min exposure to ATP, but was delayed progressively with increasing concentrations of ATP^{4-} to 6.2 ± 1.7 min (mean $\pm$ s.d., $n = 5$) at 1.11 mM. Maximal motility was slightly decreased at 0.71 and 1.11 mM- ATP^{4-} (58.4 ± 6.5 and $61.4 \pm 7.5\%$) when compared to values at 0.08 and 0.36 mM (77.0 ± 4.5 and $76.4 \pm 4.9\%$, $P < 0.05$).

Fig. 1. Effect of free ATP^{4-} on the reactivation of demembrated spermatozoa from the hamster cauda epididymidis. Calculated concentrations of ATP^{4-} were 0.08 ($\blacktriangle$), 0.36 ($\circ$), 0.71 ($\diamond$) and 1.11 ($\blacksquare$) mM as in reactivation conditions A to D (Table 1) respectively. Values are mean $\pm$ s.e.m. of 5 experiments.

In the second group of experiments, caput and cauda spermatozoa from each hamster were studied using the same conditions as in the first group. Delay in the reactivation of cauda spermatozoa was only detectable at ATP^{4-} concentrations above 0.71 mM (Table 2, A–D) while concentrations of ATP^{4-} from 0.36 to 1.11 mM exerted a greater inhibition on caput spermatozoa in the rate of attaining maximal reactivation (Wilcoxon's stratified test, $P < 0.01$).

Effect of $MgATP^{2-}$

When the concentration of ATP^{4-} was fixed at a low level of 0.2 mM, increasing $MgATP^{2-}$ concentrations from 0.9 to 1.9 mM did not affect the reactivation of cauda spermatozoa, while a further increase to 2.9 mM (6.2 mM- $MgSO_4$) caused a marked decrease in maximal percentage reactivation and delayed the initiation of motility (E–G in Table 2). ATP from two different suppliers produced the same inhibitory effect. This was not observed when the total concentration of $MgSO_4$ added remained the same (6.2 mM) and $MgATP^{2-}$ was reduced to 1 mM (condition I, Tables 1 & 2).

Table 2. Effects of increasing concentrations of ATP⁴⁻ and MgATP²⁻ on the reactivation of demembranated cauda and caput spermatozoa of hamsters (reactivation conditions as A to I in Table 1)

Reactivation condition	Maximal % reactivation		Reactivation time* (min)	
	Cauda	Caput	Cauda	Caput
mM ATP⁴⁻				
A 0.08	—	49.0 ± 6.2 ^a	—	1.2 ± 0.4 ^a
B 0.36	72.9 ± 5.9 ^a	60.3 ± 2.0 ^b	1.2 ± 0.4 ^a	2.4 ± 0.5 ^b
C 0.71	68.3 ± 9.7 ^a	47.8 ± 3.2 ^a	3.0 ± 0.6 ^b	4.0 ± 0.6 ^c
D 1.11	48.9 ± 9.5 ^b	28.3 ± 9.5 ^c	3.4 ± 1.0 ^b	5.2 ± 0.4 ^d
mM MgATP²⁻				
E 0.91	84.0 ± 5.6 ^c	66.1 ± 8.4 ^d	1.4 ± 0.6 ^c	2.0 ± 0.7 ^e
F 1.89	83.1 ± 8.6 ^c	48.3 ± 6.4 ^e	1.6 ± 0.6 ^c	3.5 ± 0.8 ^f
G 2.88	33.0 ± 19.1 ^d	4.4 ± 2.4 ^f	5.7 ± 2.7 ^d	6.3 ± 1.0 ^f
H 1.02	—	45.5 ± 6.4 ^c	—	1.6 ± 0.6 ^c
I 1.05	75.5 ± 7.3 ^c	—	1.2 ± 0.5 ^c	—

Values are mean ± s.d. of 5 experiments.

*Time required to achieve over 75% maximal reactivation after exposure to ATP; minimal time detectable was 1 min (see 'Methods').

Within columns and conditions A–D, and E–I, values with different superscript letters are significantly different ($P < 0.05$).

Table 3. Treatments affecting the inhibitory actions of 1.1 mM-free ATP⁴⁻ and 2.9 mM-MgATP²⁻ on the reactivation of demembranated spermatozoa from the hamster cauda epididymidis

Treatment	1.1 mM-ATP ⁴⁻			2.9 mM-MgATP ²⁻		
	% motility†	Min‡	n	% motility†	Min‡	n
(a) None*	58.0 ± 9.2 ^a	5.5 ± 1.4 ^a	9	33.0 ± 19.1 ^a	6.0 ± 2.8 ^a	5
(b) 1 min preincubation	50.4 ± 13.4 ^a	2.9 ± 1.1 ^b	9	15.7 ± 10.7 ^a	3.4 ± 1.8 ^b	5
(c) 2 min preincubation	58.1 ± 7.8 ^a	1.3 ± 0.5 ^c	7	24.3 ± 16.2 ^a	3.2 ± 1.1 ^b	5
(d) + trypsin	73.3 ± 4.7 ^b	1.0 ± 0.0 ^c	4	79.1 ± 7.6 ^b	1.0 ± 0.0 ^c	5
(e) + cAMP	83.9 ± 2.5 ^b	2.8 ± 1.1 ^b	9	59.2 ± 10.5 ^b	5.5 ± 2.4 ^d	4
(f) 1 min preincubation then + cAMP	82.0 ± 5.2 ^b	1.3 ± 0.7 ^c	7			
(g) 2 min preincubation in trypsin inhibitor	38.3 ± 9.7 ^c	2.8 ± 0.7 ^b	6			

Values are mean ± s.d.

†Maximal % motility during reactivation.

‡Time to achieve > 75% maximal reactivation after exposure to ATP.

*Control conditions for the study of factors affecting inhibitory actions of ATP⁴⁻ and MgATP²⁻ were as described in Table 1, D and G, respectively.

Values within columns with different superscript letters are significantly different ($P < 0.05$).

On the other hand, an increase in MgATP²⁻ concentration from 0.9 to 1.9 mM did delay the reactivation of caput spermatozoa, which was almost completely suppressed at 2.9 mM. When MgATP²⁻ concentration was again maintained at 1 mM, free Mg²⁺ at a concentration equal to that in the above inhibitory condition had no effect on the initiation of motility although it slightly decreased the maximal percentage reactivation (Table 2, E & H). A slight decrease in percentage motility of caput spermatozoa by high concentration of Mg²⁺ was also shown by reactivation conditions A and B (Table 2).

Fig. 2. Light micrographs of spermatozoa from the hamster cauda epididymidis in the demembration-reativation medium: (a) without the addition of ATP when the immotile sperm tails exhibited waves of slight curvatures; (b) in the presence of 1.1 mM-ATP^{4-} (condition D in Table 1) when the whole or proximal two-thirds of the tail coiled in a large-angle P bend; or (c) $2.9 \text{ mM-MgATP}^{2-}$ (condition G in Table 1) when coiling occurred in a direction opposite to that in (b), i.e. in R bends, before the initiation of regular motility. $\times 170$.

Factors affecting the inhibitory action of ATP on the reactivation of cauda spermatozoa

A brief preincubation of cauda spermatozoa in Solution R for 1 and 2 min before the addition of ATP resulted in a stepwise recovery of the time course of reactivation in the presence of 1.1 mM-ATP^{4-} , and the inhibition of $2.9 \text{ mM-MgATP}^{2-}$ (i.e. 1 mM-MgATP^{2-} in excess of its optimal concentration of 1.9 mM) was reversed but to a lesser extent (Table 3, treatments a–c). The presence of trypsin inhibitor in the medium only partly blocked the effect of preincubation. Trypsin, however, allowed immediate maximal reactivation involving a higher percentage of motile spermatozoa

Table 4. Number of cauda epididymal spermatozoa arrested in coils attaining or exceeding one revolution in the demembration–reactivation medium containing 1.1 mM-ATP⁴⁻ (A) or 2.9 mM-MgATP²⁻ (B), expressed as percentage of immotile spermatozoa during a 2-min period before achieving maximal % motility

		No treatment	1 min preincubation	+cAMP
(A)	All R-bend coils (<i>n</i> = 9)	19.5 ± 5.1	27.7 ± 10.5	81.4 ± 14.4*
(B)	All P-bend coils (<i>n</i> = 5)	25.1 ± 8.5	47.9 ± 8.0*	34.9 ± 11.0

Values are mean ± s.d.

*Significantly different from no-treatment group (*P* < 0.01).

than in its absence, although the duration of reactivated motility declined rapidly as described previously (Yeung, 1986).

Inhibition by 1.1 mM-ATP⁴⁻ was largely overcome in the presence of 100 µM-cAMP, but inhibition by 2.9 mM-MgATP²⁻ in the initiation of reactivation persisted despite a partial recovery in the maximal percentage motility (Table 3, treatment e).

Flagellar waveforms under ATP inhibition

Most cauda spermatozoa in the demembration and reactivation solution in the absence of ATP were immobilized with a slightly curved flagellum (Fig. 2a) as previously described (Yeung & Woolley, 1983). Before reactivation occurred, immotile spermatozoa in the presence of an inhibitory concentration of ATP⁴⁻ coiled into a large reverse (R) bend with a bend-angle reaching 360° or more (the terminology of P and R bends is that defined by Woolley, 1977). This R bend comprised the whole flagellum except when there was a short principal (P) bend at the neck and occasionally a small sharp distal P bend which failed to propagate off the tip of the tail (Fig. 2b). This feature of 'sperm coils' was more marked when the medium contained cAMP (Table 4A). When these spermatozoa first became motile, the middle region of the tail straightened and beat irregularly. These intermittent bends initially did not propagate to the tip of the flagellum where the sharp R bend curvature was retained. In about 0.5 min the whole flagellum was then recruited in regular and vigorous beating. Reactivated spermatozoa developed swimming patterns similar to those in optimal reactivation conditions with frequent display of a non-space-gaining motion referred to as 'looping' (Yeung, 1984).

Caput spermatozoa also exhibited R bends in response to increased concentrations of ATP⁴⁻ before motility was reinitiated. However, initial flagellation did not occur at mid-tail as in cauda spermatozoa, but only involved the distal half of the flagellum. The mid-piece remained stiff and did not flagellate with the principal piece under all reaction conditions.

In contrast to the effect of ATP⁴⁻, inhibition of reactivation by MgATP²⁻ induced the formation of 'sperm coils' in the opposite sense with respect to the hook of the head, resulting in a P bend. In cauda and caput spermatozoa, the mid-piece gradually curved into this P bend, accompanied by slow propagation of the original R bend towards the tip of the flagellum, until the whole tail formed a coil often exceeding 360° (Fig. 2c). This waveform persisted for a few minutes until the distal half of the tail began flagellation when reactivation started. Many coiled spermatozoa failed to develop beat cycles throughout the period of observation (up to 12 min). Preincubation enhanced the curvature of such P bend arrest (Table 4B). It was uncertain whether cAMP also enhanced the curvature because the arrest was brief, such that curvature might not have developed to the maximum before flagellation was initiated.

In the presence of trypsin, reactivation with the inhibitory concentration of either ATP⁴⁻ or MgATP²⁻ was immediate and no sperm coils appeared, except occasionally in preparations with

2.9 mM-MgATP²⁻ in which motile spermatozoa retained a small sharp rigid P bend near the base of the flagellum.

Discussion

The substrate for dynein ATPase is MgATP²⁻. Alteration of the concentrations of total ATP in the reactivation medium should result in changes in the concentrations of MgATP²⁻, ATP⁴⁻ and Mg²⁺, all of which may be involved in the regulation of flagellar movement (Okuno & Brokaw, 1979). Study of the individual effects of these chemical species on sperm motility is complicated by the dissociation equilibrium which makes it impossible to adjust the concentrations of one independently of the other two. In this study, the levels of total ATP throughout the incubation should be stable since hydrolysis by the low concentrations of spermatozoa, even when motile, is estimated from literature values to be less than 1%. Suppression of reactivation by increasing the concentration of MgATP²⁻ at a constant and low concentration of ATP⁴⁻ was accompanied by an increase in Mg²⁺ concentrations. Although increasing concentrations of Mg²⁺ could decrease sea-urchin sperm flagellation (Okuno & Brokaw, 1979), neither the highest concentration of total MgSO₄ nor that of Mg²⁺ tested was responsible for such effects when MgATP²⁻ was kept at low concentrations. Increase in the concentrations of ATP⁴⁻ in the presence of a relatively constant level of MgATP²⁻ also led to a progressive delay in the initiation of motility in demembranated caput and cauda spermatozoa. These findings agree with the previous results (Yeung, 1986) in which temporary inhibition of reactivation was obtained using 3 mM-ATP and 4 mM-MgSO₄, which should produce 0.51 mM-ATP⁴⁻ and 2.5 mM-MgATP²⁻.

Although dynein ATPase extracted from sea-urchin spermatozoa is inhibited by free ATP (Hayashi, 1976), neither ATP⁴⁻ nor MgATP²⁻ in concentrations used in the present study on hamster spermatozoa appeared to exert their inhibitory action on motility directly on the active sliding of microtubules, since maximal reactivation occurred without delay in the presence of trypsin suggesting that the mechanisms involved are trypsin sensitive. However, these two inhibitory mechanisms are not the same, as indicated by the different characteristic flagellar waveforms induced and the ability of cAMP to overcome the blockade, particularly in the initiation of reactivation, by ATP⁴⁻ but not by MgATP²⁻.

Regulation of sperm motility by cyclic AMP is well known (see reviews by Garbers & Kopf, 1980; Tash & Means, 1983). Cyclic AMP is required for immediate reactivation of demembranated mature rat spermatozoa (Fentie & Lindemann, 1978; Treetipsatit & Chulavatnatol, 1982) and can enhance the motility of demembranated immature spermatozoa from hamsters (Yeung, 1984; Ishijima & Mohri, 1985). Activation of sperm flagellation involves the cAMP-dependent phosphorylation of a specific axonemal protein, axokinin (Tash *et al.*, 1984, 1986). The action of ATP⁴⁻ described here could be via the reduction of endogenous adenylate cyclase activity and the resultant reduced production of cAMP, although it has been demonstrated by enzyme-kinetic analysis that free ATP⁴⁻ is not a strong inhibitor of bovine sperm adenylate cyclase if at all (Brown & Casillas, 1986). Another possible indirect reduction in this enzyme activity by increasing ATP⁴⁻ concentrations could result from the concomitant decrease in the concentrations of Mg²⁺, a stimulator for the enzyme. However, the delay in the initiation of reactivation was not as completely abolished by cAMP alone as by cAMP added after 1 min preincubation of the sperm preparation, suggesting that some other factors are involved, which may include the endogenous specific protease activity that regulates sperm motility (de Lamirande & Gagnon, 1986). This unknown factor was not specifically sensitive to soybean trypsin inhibitor because the presence of the latter did not completely suppress the enhancing effect of preincubation.

The mechanism of the inhibitory effect of MgATP²⁻ is unclear. Failure of cAMP to completely overcome this inhibition suggests that the blockade of the initiation of bend cycles occurs before the step regulated by cAMP.

The present observations for hamster spermatozoa are probably the manifestation of some general features of flagella and cilia. Superoptimal concentrations of ATP can arrest sliding of microtubules in *Tetrahymena* cilia (Warner & Zanetti, 1980) and induce quiescence in sea-urchin spermatozoa (Brokaw, 1984). These phenomena, as well as the inhibitory effects of other factors on flagellar movement including calcium and lithium ions (Gibbons & Gibbons, 1980, 1984), are also abolished by gentle trypsin digestion. Further study of the induction of formation of 'sperm coils' in opposite senses of ATP^{4-} and MgATP^{2-} and the subsequent initiation of beat cycles may contribute to the understanding of bend initiation and the 'switch-point' mechanism of active sliding of microtubule postulated as the basis for bending cycles in cilia and flagella (see review by Satir, 1984).

While further investigation is required to elucidate the inhibitory mechanisms of these two ATP species, the present findings emphasize the subtlety of the regulation of sperm motility through ATP concentration as well as its various ionic forms.

I thank Professor E. Nieschlag and Dr T. G. Cooper for helpful comments on the manuscript.

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Received 30 January 1987