EFFECTS OF VANADATE, Mg²⁺ AND ELECTRIC CURRENT INJECTION ON THE STIFFNESS OF IMPALED BULL SPERMATOZOA

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SUMMARY

Measurements were made of the rigor stiffness of impaled bull sperm flagella. Vanadate-free ATP was found to have a stronger plasticizing action than vanadate-contaminated ATP. Trace amounts of Mg²⁺ were found to be necessary to relieve the rigor condition by ATP. During the injection of negative electric current into the sperm head, the flagella stiffened, presumably by depletion of Mg²⁺ from the flagellar interior.

INTRODUCTION

The phenomenon of 'rigor' has recently been described in the flagella of impaled bull sperm (Lindemann, Rudd & Rikmenspoel, 1973) and of demembranated sea-urchin sperm (Gibbons & Gibbons, 1974, 1978). In both species the stiffening of the flagella was found to result from a deficiency of ATP. In the sea-urchin sperm flagellum the rigor has been explained as a permanent attachment of dynein cross-bridges between the axonemal fibres (Gibbons & Gibbons, 1974; Penningroth & Witman, 1978). It has been suggested by Warner (1978) that Mg²⁺ is required to effect this attachment. Bull spermatozoa have a more complicated structure than the simple axoneme of sea-urchin spermatozoa. Each axonemal tubulin fibre is accompanied by an auxiliary ‘coarse fibre’, located radially at the outside of the axonemal fibre. The whole structure is surrounded by a fibrous sheath (Fawcett, 1970). Both the coarse fibres and the fibrous sheath contribute to the stiffness of the bull sperm flagellum (Phillips, 1972; Rikmenspoel, 1965). This greater stiffness has made it possible to measure the stiffness of the bull sperm flagella in a direct way (Lindemann et al. 1973).

In the experiments in which the method for the direct stiffness measurements was developed, ATP was used that has since been shown to be contaminated with vanadate (Beauge & Glynn, 1977; Josephson & Cantley, 1977). This ATP was unable to maintain normal flagellar motion in impaled or in demembranated bull sperm (Lindemann & Gibbons, 1975; Lindemann & Rikmenspoel, 1972). Recently, normal flagellar activity has been obtained in impaled and in demembranated bull sperm by means of ATP from a new source and presumably with a higher purity (Rikmenspoel, Orris & O'Day, 1978; Lindemann & Gibbons, 1975). In
Fig. 1. Principle of the method of measuring flagellar stiffness. The microelectrode probe is used to move the sperm flagellum out of the equilibrium position $E$. The return to position $E$ is shown at various time intervals. The time origin $(t = 0)$ has to be taken well after the release of the flagellar tip from the probe. For further details see Lindemann et al. (1973).

In this paper, the results are presented of stiffness measurements on bull sperm flagella as a function of the concentrations of pure ATP, of pure ATP with vanadate added, and of Mg$^{2+}$; and when electric current was injected into the sperm.

**MATERIALS AND METHODS**

**Sperm preparations**

Bull spermatozoa were generously supplied by the Eastern Artificial Insemination Cooperative at Ithaca, New York. After collection, the semen was diluted 5 times in a clear egg-yolk diluent as described previously (Rikmenspoel, 1957), cooled to 4 °C and transported to the laboratory.

The sperm were washed twice in basic suspension medium (BM), as described below. For the experiments 1 drop of the twice-washed sperm was added to 1 ml of BM. Additions to this final suspension were made as desired.

Our washing procedure resulted in an effective dilution of the original medium for the sperm by a factor of $10^4$. Since the egg-yolk and semen-plasma mixture in which the sperm were received contained approximately 1 mM-Mg$^{2+}$, the concentration of Mg$^{2+}$ in the final experimental suspension, if no Mg$^{2+}$ additions were made, was of the order of 0.1 to 1 μM.

**Solutions and chemicals**

The basic suspension medium (BM) for the sperm preparations consisted of 72 mM-KCl, 160 mM-sucrose, 2 mM-Na lactate and 2% of 0.1 M-NaH$_2$PO$_4$ buffer in quartz-double-distilled water. The pH was adjusted to 7.4. In this medium, with the appropriate additions of Mg$^{2+}$ and ATP, a normal motility of bull sperm flagella can be maintained after impalement (Rikmenspoel et al. 1978).

Disodium ATP was obtained from Boehringer Mannheim Co. (Indianapolis, IN). Aliquots of 1 ml of 0.1 M-ATP at pH 7.4 were stored frozen and thawed just prior to use.

Sodium metavanadate (NaVO$_4$), obtained from Fisher Scientific Co. (Pittsburgh, PA), was added when desired from a 0.4 mM stock solution, which had been adjusted to pH 7.4.

Mg$^{2+}$ was added from a 0.1 M solution of MgSO$_4$. EDTA and EGTA were obtained from Sigma Chemical Co. (St Louis, MO).

**Stiffness measurements**

During experiments the bull sperm were observed under a Zeiss 40 x water-immersion microscope objective. After the washing procedure described above most sperm were stuck by the head to the microscope slide, while the flagella remained motile. The sperm were
Stiffness of bull spermatozoa

Probe

Current electrode

Fig. 2. Enlargement of a frame of 16 mm film illustrating the measurement of flagellar stiffness during the injection of electric current by means of a second microelectrode.

desactivated by impaling the head with a glass microelectrode, as previously described (Lindemann & Rikmenspoel, 1971). In all experiments either Mg$^{2+}$ or ATP was deleted from the medium, to avoid post-impalement flagellar activity. It should be noted that after impalement the interior of the sperm is in free diffusional equilibrium with the outside medium (Lindemann & Rikmenspoel, 1971; Rikmenspoel et al. 1978). The concentration of the components of the medium was thus approximately the same at the interior and the exterior of the impaled sperm.

After impalement the microelectrode was withdrawn from the sperm head. It was then used as a probe to displace the sperm flagellum out of its more or less straight equilibrium position, as illustrated in Fig. 1. When the flagellum was released from the probe, it returned to the equilibrium position. The characteristic time, $\tau_e$, in which the flagellum returns to its equilibrium position is inversely proportional to the stiffness, $IE$, of the flagellum (see Discussion for the value of $A$):

$$IE = A/\tau_e.$$  

The characteristic relaxation times, $\tau_e$, defined as the time in which the deviation from the equilibrium position decreases by a factor $1/e$, was measured from moving films, as previously described (Lindemann et al. 1973).

A full discussion of the above method of measuring flagellar stiffness has been published (Lindemann et al. 1973). In Results the experimental data will be reported as the value of the primary measured constant, $\tau_e$.

**Current injection**

Recently, experiments from this laboratory have been reported (O'Day & Rikmenspoel, 1979), in which the flagellar activity of bull sperm was controlled by electrical current through a microelectrode impaling the head of a sperm. Full details of the validation of the methods, and the results of these electrical control experiments are given in the paper referred to above.

It proved quite possible to perform stiffness measurements on impaled bull sperm while electrical current was being injected. This required working with 2 microelectrodes. One microelectrode impaled the head and was employed for current injection. The second microelectrode was used to manipulate the flagellar tip, as described above. Fig. 2 illustrates the procedure of the stiffness measurement on an impaled bull sperm during the injection of electric current.

**RESULTS**

**Effects of ATP and vanadate**

Table 1 shows the value of $\tau_e$ measured on bull sperm in BM with different concentrations of ATP, but with no Mg$^{2+}$ added. The value of $\tau_e = 33 \pm 6$ ms agrees with the previously reported value of $36 \pm 10$ ms (Lindemann et al. 1973) for the case where [ATP] = 0. At the higher ATP concentrations (3 and 6 mm) the relaxation
Table 1. Characteristic relaxation time $\tau_c$ for the return of bull sperm flagella to the elastic equilibrium position as a function of the ATP and vanadate concentrations

<table>
<thead>
<tr>
<th>Addition to medium</th>
<th>No. of sperm</th>
<th>$\tau_c$ (av. ± S.D.) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP (mM) Vanadate (µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 —</td>
<td>4</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>2 —</td>
<td>4</td>
<td>480 ± 150</td>
</tr>
<tr>
<td>3 —</td>
<td>6</td>
<td>540 ± 310</td>
</tr>
<tr>
<td>6 0</td>
<td>6</td>
<td>1560 ± 880</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>300 ± 180</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>200 ± 110</td>
</tr>
<tr>
<td>10 1</td>
<td>5</td>
<td>390 ± 230</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>370 ± 140</td>
</tr>
</tbody>
</table>

No Mg$^{2+}$ was added to the external medium. For values of $\tau_c > 100$ ms the last digit in this table and Tables 2, 3 and 4 was rounded off.

The present, pure ATP thus has a greater plasticizing action than the impure ATP. This was confirmed by the addition of vanadate to the external medium, which significantly reduced the plasticizing action of the pure ATP (Table 1). The average values of $\tau_c$ obtained with 5 and 10 mM of the impure ATP had previously been found to be 213 ± 84 ms and 462 ± 30 ms, respectively (Lindemann et al. 1973). Table 1 shows that in the presence of 1 µM-vanadate, the $\tau_c$ values for 6 and 10 mM pure ATP approximate those of the contaminated ATP. Therefore, 1 mol of the previously used impure ATP contained about $10^{-4}$ mol of vanadate contaminant.

In addition, in separate experiments it was found that 1 µM of vanadate eliminated the flagellar motion of impaled sperm that is normally maintained in the presence of Mg$^{2+}$.

It can be seen in Table 1 that the spread in the measured values becomes larger for the longer relaxation times. This is probably due at least in part to the small restoring force in the very flaccid flagella, which makes the return to the equilibrium position more sensitive to small obstructions in the fluid, to unevenness of the adjacent slide surface etc. The differences in $\tau_c$, and therefore in the stiffness, under the various conditions, are so large, however, that the effects are unmistakable.

Effects of Mg$^{2+}$

Mg$^{2+}$ itself was found to have no influence on the stiffness of the bull sperm flagella. Table 2 shows that, up to a concentration of 3 mM, the addition of Mg$^{2+}$ without ATP results in complete rigor of the flagella upon impalement.

Table 3 shows that when EDTA was added to a medium containing 3 mM-ATP but no Mg$^{2+}$, the bull sperm flagella stiffened. Complete rigor was attained at an EDTA concentration of 1.5 mM. EGTA, however, has no effect on the flagellar stiffness up to a concentration of 1.5 mM. It was noted above in the first section of
Table 2. Characteristic relaxation time $\tau_c$ for bull sperm flagella as a function of the external Mg$^{2+}$ concentration

<table>
<thead>
<tr>
<th>Mg$^{2+}$ concn (mM)</th>
<th>No. of sperm</th>
<th>$\tau_c$ (av. ± S.D.) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>35 ± 7</td>
</tr>
</tbody>
</table>

No ATP was present.

Materials and methods that our washing procedure leaves a concentration of Mg$^{2+}$ in the final experimental suspension of $\sim 1$ $\mu$M. Since EDTA binds strongly to Mg$^{2+}$, and EGTA binds weakly (Dawson, Elliott, Elliott & Jones, 1969; Donaldson & Rerrick, 1975), the results in Table 3 indicate that a trace amount of Mg$^{2+}$ is needed for ATP to perform its plasticizing role.

Electric current injection

Bull sperm in BM with 6 mM-ATP but no Mg$^{2+}$ added were impaled with a micro-electrode. The stiffness of the flagella was measured, as described in Materials and methods, while direct electric current was passed through the impaling electrode. Table 4 shows that the relaxation time, $\tau_c$, gradually decreased (and therefore the stiffness increased) as more electric current was injected. Complete rigor was developed in the flagella at an injected current of $\sim 2 \mu$A.

A number of spermatozoa in BM, with either 3 or 6 mM-ATP and no Mg$^{2+}$ added, were brought into complete rigor by the injection of $\sim 1-8 \mu$A of electric current. Upon cessation of the injection of current the flagella returned nearly to the original flaccidity. The measured average of $\tau_c$ after the injection of current was $0.8 \pm 0.3$ (av. ± S.D.) of that before the start of the injection of current.

In a separate experiment, spermatozoa in BM, with 3 mM-ATP and 0.2 mM-EDTA but no Mg$^{2+}$ added, were injected with $\sim 1.8 \mu$A of electric current. Upon cessation of the injection of current, the rigor condition remained. The value of $\tau_c$ measured several minutes after cessation of the electric current was $33 \pm 8$ ms (av. ± S.D.). This experiment tends to show that the injection of electrical current causes the release of Mg$^{2+}$ from a binding site. The released Mg$^{2+}$ is then chelated by EDTA.

DISCUSSION

The pure ATP, used in the present experiments, reduced the stiffness of the bull sperm flagella to lower values than the presumably contaminated ATP used previously. The value of $\tau_c$ at 6 mM-ATP reported in this paper ($\tau_c \approx 1.6$ s) is approximately three times that found previously with 10 mM of impure ATP ($\tau_c \approx 0.5$ s). The stiffness of the flagella at a concentration of pure ATP of 6 mM is thus three times lower than that at 10 mM of vanadate-contaminated ATP. That the increased stiffness
Table 3. Characteristic relaxation time \( \tau_c \) for impaled bull sperm with 3 mM-ATP and no \( \text{Mg}^{2+} \) added externally, at various concentrations of EDTA and EGTA

<table>
<thead>
<tr>
<th>Chelator</th>
<th>Concentration (mM)</th>
<th>No. of sperm</th>
<th>( \tau_c ) (av. ± S.D.) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>6</td>
<td>540 ± 310</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.5</td>
<td>4</td>
<td>270 ± 90</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4</td>
<td>77 ± 55</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>6</td>
<td>36 ± 8</td>
</tr>
<tr>
<td>EGTA</td>
<td>1.5</td>
<td>6</td>
<td>560 ± 370</td>
</tr>
</tbody>
</table>

Table 4. Relaxation time \( \tau_c \) for impaled bull sperm, in BM, with 6 mM-ATP but no \( \text{Mg}^{2+} \) added, during the injection of negative electric current through the impaling electrode

<table>
<thead>
<tr>
<th>Electric current (µA)</th>
<th>No. of sperm</th>
<th>( \tau_c ) (av. ± S.D.) (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>1640 ± 910</td>
</tr>
<tr>
<td>-0.1</td>
<td>4</td>
<td>1490 ± 480</td>
</tr>
<tr>
<td>-0.3</td>
<td>4</td>
<td>860 ± 520</td>
</tr>
<tr>
<td>-1</td>
<td>6</td>
<td>41 ± 35</td>
</tr>
<tr>
<td>-2</td>
<td>7</td>
<td>31 ± 15</td>
</tr>
</tbody>
</table>

found previously was due to a vanadate contaminant in the ATP is supported by the present experiments, in which the stiffness values obtained with pure ATP are markedly increased upon the external addition of vanadate to the medium. Indeed, 1 µM-vanadate caused a fivefold decrease in the \( \tau_c \) value for pure ATP, thereby increasing the stiffness to the range of that reported previously for the same concentration of contaminated ATP.

Our present observations that µM concentrations of vanadate inhibit the post impalement motility of bull sperm flagella (in the presence of \( \text{Mg}^{2+} \) and ATP or ADP) parallel the results of Gibbons et al. (1978), who found that similar concentrations of vanadate inhibited the reactivation of motility in demembranated sea-urchin flagella.

It is possible that concentrations of pure ATP higher than 6 mM would lead to a further decrease of stiffness. It does not seem meaningful, however, to attempt these measurements with the method used here, because the errors in the value of \( \tau_c \) can be expected to become excessively large (compare Table 1).

The reported values of \( \tau_c \) can be transformed into absolute values for the stiffness, \( IE \), of the flagella at the midpiece–flagellar junction by means of equation (1) above. In the paper in which the method for the stiffness measurement was originally discussed (Lindemann et al. 1973), the value of the constant \( A \) in equation (1) was given as \( A = 1.06 \times 10^{-17} \) N cm² s⁻¹, if the flagellum was approximated as a straight cylinder. Corrections for the taper of the flagellum, and uncertainty in the exact value for the fluid drag coefficient in the present case led to a value for \( A \) of 0.9 to 1.9 \( \times 10^{-17} \)
N cm² s. The value of $\tau_c = 1.6$ s presently found at a 6 mM-ATP concentration thus leads to a stiffness, $IE = A/\tau_c = 0.6$ to $1.2 \times 10^{-17}$ N cm². This value is compatible with the value, $IE = 1.8 \times 10^{-17}$ N cm², derived previously from dynamic treatments of motile bull sperm flagella (Rikmenspoel, 1965, 1978). It should be kept in mind, however, that there is no assurance that an identical value for the stiffness should apply to a fully relaxed non-motile flagellum as to an active, motile one. For bull sperm flagella in complete rigor ($\tau_c = 33$ ms) the value for the stiffness is $IE = 3$ to $6 \times 10^{-18}$ N cm².

Okuno (1980) and Okuno & Hiramoto (1979) have recently reported values for the stiffness of the sea-urchin sperm axoneme of $1.1 \times 10^{-16}$ N cm² when the axonemes were in rigor, and of $1 \times 10^{-17}$ N cm² when the axonemes were fully relaxed. These results indicate that an appreciable fraction of the stiffness of the bull sperm flagella may originate in its axonemal structure. It should be noted, however, that the static measurement method employed by Okuno (1980) yields values for the stiffness of the sea-urchin axoneme, an order of magnitude higher than those obtained from dynamic analysis of moving sea-urchin sperm or ciliary axonemes (Rikmenspoel, 1966, 1978; Lindemann, 1975).

The magnesium ion, while not by itself causing a relief from rigor of the bull sperm flagella (Table 2), is needed in trace quantities to enable ATP to reduce the flagellar rigor. This is shown by the fact that in the presence of EDTA the rigor was not relieved by ATP, but in the presence of EGTA it was. The concentration of EDTA (1-1.5 mM) needed to cause more or less complete rigor in the presence of ATP is more than 3 orders of magnitude larger, however, than the concentration of Mg²⁺ (0.1-1 μM) left in the medium after our washing procedure. It should be noted that in all experiments described in this paper the sperm had been impaled by a microelectrode. Through the hole made by the impalement the interior of the sperm was then in free diffusional equilibrium with the external medium (Lindemann & Rikmenspoel, 1971; Rikmenspoel et al. 1978). The EDTA concentration in the interior of the sperm flagella was thus comparable to that of the external medium. The high concentration of EDTA needed to cause rigor indicates that the Mg²⁺ that is functional in mediating the plasticizing action of ATP is strongly bound at a site in the flagella with a binding constant comparable to or higher than that of EDTA to Mg²⁺, i.e. $6 \times 10^5$ (Dawson, Elliott, Elliott & Jones, 1969; Donaldson & Rerrick, 1975).

The plasticizing action of ATP occurs at concentrations in the mM range. This means that the Mg-ATP complex in solution, which has a maximum concentration range of $0.1-1$ μM in our experiments, is not the agent directly responsible for the relief of the flagellar rigor. This result also points to the fact that Mg²⁺ is bound to a site in the flagella, to make this site accessible to ATP. The location of this site cannot be established from the present experiments. One can speculate, however, that it is on the coarse, auxiliary fibres in the bull sperm flagella (Fawcett, 1970) since these fibres do appear to have a role in providing stiffness in the flagella (Phillips, 1972).

During the injection of electric current the stiffness of the flagella of the bull sperm increased (Table 4). This should not be interpreted as indicating that the ATP concentration inside the flagella had decreased during injection of current. Firstly, no
mechanism is readily conceivable for removing ATP from the motionless flagella by injection of electric current. Secondly, at an injected current of \(-0.3 \mu A\), the stiffness of the flagella (Table 4) is the same as that corresponding to an ATP concentration, without injection of current, of approximately 4 mM, as can be seen from interpolation of Table 1. A reduction of the ATP concentration from 6 mM to 4 mM does not affect the motility of a bull sperm flagella (Rikmenspoel et al. 1978). The injection of \(-0.3 \mu A\) of electric current does, however, cause a severe reduction of flagellar activity (O'Day & Rikmenspoel, 1979) in a motile sperm. We should conclude, therefore, that the increased stiffness during injection of electric current is not caused by a lowering of the ATP level in the flagella.

In the paper describing the experiments on the control of bull sperm motility by the injection of electric current, it was concluded (O'Day & Rikmenspoel, 1979) that the reduction of flagellar activity during injection of current was due to removal of Mg\(^{2+}\) from the inside of the flagella. This is supported by the present observations that the bull sperm flagella stiffen during injection of current, but on cessation of the current resume approximately normal stiffness only in the absence of EDTA. Any Mg\(^{2+}\) loosened from a site in the sperm flagellum during injection of current is of course chelated by EDTA present at the concentration (0.2 mM) used in our experiments.

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