The flagellar beat of rat sperm is organized by the interaction of two functionally distinct populations of dynein bridges with a stable central axonemal partition

CHARLES B. LINDEMANN, ANGELA ORLANDO and KATHLEEN S. KANOUS

Department of Biological Science, Oakland University, Rochester, MI 48309-4401, USA

Summary

Two distinct patterns of microtubular sliding were observed in rat sperm flagellar axonemes. The particular pattern of sliding was determined by the extraction conditions used to prepare the sperm for axoneme disintegration. Sperm prepared by incubating concentrated suspensions of Triton X-100-extracted sperm at pH 9.0 disintegrated by extruding the doublets and outer dense fibers numbered 4 through 7 in response to Mg-ATP. Sperm prepared by incubating motile Triton X-100-extracted models at 37°C for 1 to 3 hours extruded doublets and outer dense fibers 9, 1 and 2. Axonemes disintegrated by both regimens tended to have doublets 3 and 8 (with their corresponding outer dense fibers), as well as the central pair, in place. In numerous instances, the 3-central-8 complex with outer dense fibers 3 and 8 could be found isolated in midpiece sections prepared from both methods. The 3-central-8 partition was also sometimes seen in isolation in cross-sections of the principal piece where it remained attached to the fibrous sheath. The flagellar remnant produced by extrusion of fibers 4 through 7 under high pH conditions was generally straight or randomly curved. In contrast, the flagellar remnant produced by extrusion of the 9-1-2 bundle of fibers was most often curved into a hook in the midpiece region. While the hook-like configuration was not Ca²⁺-dependent, it may be based on a related mechanism. The sliding of the 9-1-2 group of fibers is a consequence of dynein-tubulin sliding between the 2 and 3 doublets. This sliding pattern appears to be preferentially activated in the motile sperm models in EGTA, but seldom if ever produced sliding in the high-pH-extracted models. We conclude that the 3-central pair-8 complex and associated outer dense fibers form an I-beam-like partition that does not participate in sliding, but acts as a structural foundation for organizing a planar beat. In addition, it is clear that preferential activation of certain dynein arms can be evoked, depending on the treatment regimen employed. This shows definitively that the types of microtubule sliding in the two bend directions are not identical.

Key words: dynein, outer dense fibers, microtubules, calcium, microtubule sliding, flagellar beating, central pair microtubules.

Introduction

Both ciliary and flagellar motion are driven by forces produced via the dynein-tubulin interaction (Satir, 1979). This interactive mechanism produces a longitudinal displacement of adjacent microtubules relative to each other, a process that has been observed directly in intact (Satir, 1968) and partially digested axonemes (Summers and Gibbons, 1971, 1973; Lindemann and Gibbons, 1975). If sliding is not impeded by the structural connections of the nexin and radial spokes, the dynein-tubulin interaction produces a continuous sliding of the microtubules (Summers and Gibbons, 1973). With the possible exception of the 5-6 doublets (reported to be permanently bridged), each microtubule seems capable of sliding on the next one (Summers and Gibbons, 1971, 1973). Furthermore, sliding always occurs in a polarity resulting in each doublet sliding baseward on its higher numbered neighbor (Sale and Satir, 1977).

These findings, while clear and unequivocal, do not provide much insight into the functional organization of the intact axoneme. Since all but two of the nine outer doublets are capable of sliding, the forces distorting the axoneme should generate equal bending movements in all directions, giving rise to a helical wave. Yet, in a wide range of organisms, from sea urchin sperm to large mammalian sperm, the flagellar bending waves are predominantly planar, with only a small helical component (Brokaw, 1965; Rikmenspoel, 1966; Brokaw, 1972; Phillips, 1972; Gibbons and Gibbons, 1973; Denehy et al. 1975; Wooley, 1977; Chevrier and Dacheux, 1987; Ishijima and Mohri, 1990).

The central pair is aligned ~90° to the plane of
bending. Theoretically, the additional stiffness of the central pair in one plane may favor bending in the orthogonal plane. However, being centrally located, it is unlikely that the central pair alone can contribute sufficient stiffness to reduce bending in that plane by more than 90% (as is observed in most sea urchin sperm). Alternately, mechanisms have been proposed in which the selective activation of certain doublet pairs could lead to the production of force in one predominant direction at a time (Wais-Steider and Satir, 1979). Such a scheme may be involved in the orientation of the beat (a switching hypothesis). There is evidence to substantiate the preferred activation of certain sliding pairs, on the basis of free Ca\(^{2+}\) levels (Sale, 1986; Satir and Matsuoka, 1989).

Like sea urchin sperm and other cilia and flagella, mammalian sperm also exhibit a predominantly planar beat (Rikmenspoel, 1966; Phillips, 1972; Fray et al., 1972; Fawcett, 1975; Wooley, 1977; Ishijima and Mohri, 1985), and Ca\(^{2+}\) levels influence the flagellar shape and beating pattern (Lindemann and Goltz, 1988). Specifically, high free Ca\(^{2+}\) (> 3 x 10\(^{-6}\)) imparts a hook-like beat (a switching hypothesis). There is evidence to substantiate the preferred activation of certain sliding pairs, on the basis of free Ca\(^{2+}\) levels (Sale, 1986; Satir and Matsuoka, 1989).

Materials and methods

Collection of sperm

Rat sperm were removed from the cauda epididymis of male Sprague-Dawley retired breeders obtained from Charles Rivers and killed by CO\(_2\) asphyxia. Each epididymis was minced, and sperm were gently expressed into 4 ml of the appropriate solution (either 0.9% NaCl for the low temperature/pH 9 method, or a citrate buffer containing 0.097 M citrate, 5 mM MgSO\(_4\), and 2 mM fructose at pH 7.4 for the high temperature/pH 7.8 method). The sperm suspensions were stored at room temperature prior to their use.

High temperature/pH 7.8 (HT/pH7.8) method

Rat sperm were demembranated by transferring 30 µl of sperm suspension in citrate buffer into 3 ml of reactivation mixture in a cell culture dish. The reactivation mixture contained 0.024 M potassium glutamate, 0.132 M sucrose, 0.02 M Tris-HCl, 1 mM dithiothreitol (DTT), 2 mM MgSO\(_4\), 0.5 mM EGTA and 0.1% Triton X-100 at pH 7.8. In some experiments, an additional preparation was included using the above reactivation mixture with the addition of 1 mM CaCl\(_2\) to induce flagellar hooks. After initial observation for the absence of motility, 0.3 mM ATP was added, followed by 3 µM cAMP. The demembranated sperm preparations were examined for activity and incubated at 37°C. These models were allowed to reactivate for anywhere from 1 to 3 hours, with periodic inspection to determine whether doublet-ODF sliding had occurred. Flagellar curvature data were collected from video recordings.

Low temperature/pH 9 (LT/pH9) method

High pH and proteolytic digestion have been used to remove the mitochondrial remnant from Triton X-100-extracted mammalian sperm and to permit microtubule sliding in response to MgATP (Lindemann and Gibbons, 1975; Olson and Linck, 1977). In this study, we have adopted the method of Olson and Link (1977).

Sperm suspended in 0.9% NaCl were pelleted by centrifugation for 10 minutes at 540 g. The sperm pellet was extracted in a solution containing 0.5% Triton X-100, 100 mM NaCl, 5 mM MgCl\(_2\), 2 mM DTT and 20 mM Tris-HCl buffer for two 30-minute intervals (changing to fresh solution after the first 30 minutes). These extractions were carried out at a pH of 9.0 at 4°C, and in the presence of 1 mM ATP. In some experiments, two samples were used; 1 mM CaCl\(_2\) was added to one preparation, and 0.5 mM EGTA was added to the other, to determine the effect of calcium on the sliding polarity using this method. Following Triton extraction, the sperm were washed twice by centrifugation for 10 minutes each at 540 g in a solution containing 100 mM NaCl, 5 mM MgCl\(_2\), 2 mM DTT and 20 mM Tris-HCl buffer at pH 7.8.

pH incubation

Using preparations containing rat sperm models with hooked flagella (formed either by the addition of 1 mM CaCl\(_2\) to a fresh reactivation preparation as described by Lindemann and Goltz (1988) or following ODF sliding using the HT/pH7.8 procedure), the pH was titrated to 9.0 using 1 M KOH. After
of the flagellum in uniform focus were chosen. This method
sperm. The curve of the first 50 µm of the flagellum was
curvature of the flagellum in the midpiece region of the
Videotape recordings were analyzed to estimate the radius of
with a x40 objective and a 35 mm camera.
post-fixed in 2% osmium tetroxide, washed twice in NaH2PO4
graded series of ethanol rinses. After dehydration, the pellet
with a x40 objective and a 35 mm camera.
post-fixed in 2% osmium tetroxide, washed twice in NaH2PO4
graded series of ethanol rinses. After dehydration, the pellet

Curvature determinations
Videotape recordings were analyzed to estimate the radius of
curvature of the flagellum in the midpiece region of the
sperm. The curve of the first 50 µm of the flagellum was
matched to circles of known radius. In order to ensure that the
flagellar curve was in the focal plane, only sperm with > 50 µm
of the flagellum in uniform focus were chosen. This method
allowed rapid, approximate estimations of curvature in a
larger population of cells, rather than focusing on a few,
selected cells. While the absolute values were not precise
(because the real flagellar curvature was not uniformly
circular), the assessment of the direction and relative
magnitude of the curvature was reliable. For every data point
of a single experiment, 15-20 sperm were measured. We have
successfully used this method of data collection in previous
studies (see Lindemann and Goltz, 1988; Lindemann et al.
1990).

Electron microscopy of disintegrated sperm
Sperm suspensions, obtained following either the HT/pH7.8
or the LT/pH9 method, were pelleted by centrifugation. The
pellet was fixed with 2.5% glutaraldehyde, rinsed in 10 mM
NaH2PO4 buffer and stored at 4°C overnight. The pellet was
post-fixed in 2% osmium tetroxide, washed twice in NaH2PO4
buffer, cut into 2 mm sections and dehydrated through a
graded series of ethanol rinses. After dehydration, the pellet
pieces were embedded in Poly/Bed 812. Thin sections were
cut and stained with lead and uranyl acetate, then examined
using a Philips 410-LS transmission electron microscope.

Data collection from electron microscopy sections
In addition to photographs, numerical data were collected at
the electron microscope. Sperm cross-sections were observed,
the isolated (extruded) fibers in the vicinity of each
cross-section were noted, and the associations seen between
extruded fibers were logged. Care was taken to search
through the thin-section area in a particular pattern to avoid
counting the same cross-section more than once. In addition,
at least two different preparations were observed and counted
for each set of experimental conditions to verify that the result
was not dependent on a unique sample. The data presented in
Figs 3 and 4 and Table 2 were taken from the best (cleanest
and containing the highest number of countable cross-
sections) sample available for each condition.

Results
The pattern of microtubule sliding in rat sperm was observed
and analyzed using two different methods to elicit axonemal disintegration. In one method, adopted
from the procedure developed by Olson and Linck (1977), pelleted sperm were extracted at low tempera-
ture and high pH (LT/pH9 method) to produce partially
digested Triton X-100-extracted models. After treatment,
the sperm lacked the mitochondrial sheath and
disintegrated in response to MgATP. In the absence of
MgATP, disintegration did not occur. The second
method involved incubating Triton X-100-extracted sperm models for 1 to 3 hours at 37°C after inducing
maximal motility with 3 µM cAMP, 0.3 mM ATP and
0.5 mM ethylene glycol bis(β-aminoethyl ether)-
N,N',N,N'-tetraacetic acid (EGTA) (high temperature/
pH 7.8, HT/pH7.8). This HT/pH7.8 procedure
works when motility is vigorous, but did not work as
well in the presence of Ca2+ (which substantially
decreased motility.) With the LT/pH9 method, a
consistently high percentage of sperm disintegrated in
the presence of ATP (80-90%). Using the HT/pH7.8
procedure, the percentage rarely exceeded 60%; how-
ever, sperm were often actively motile up to the time of
fiber extrusion. Furthermore, in the HT/pH7.8 regi-
men, fiber extraction depended on vigorous motility,
making it more likely that the forces produced during
coordinated flagellar bending are responsible for the
dissociation of the axoneme. In contrast, while active
dynein bridges must be present for sliding to occur in
the LT/pH9 protocol, flagellar beating was never
observed.

The disintegration of rat sperm followed a different
course under the two protocols. Following LT/pH9
extraction, doublets and ODFs from the side of the
flagellum opposite the acrosomal cap emerged as a
group (Fig. 1A). Using the HT/pH7.8 protocol, a
bundle of doublet-ODFs emerged from the neck region
on the same side as the acrosomal cap (Fig. 1B). Occasionally, a second bundle emerged from the
opposite side of the flagellum at the midpiece-principal
piece junction (Fig. 1C). The LT/pH9 method rarely
resulted in a flagellar remnant with a pronounced
curvature (hook), whereas in HT/pH7.8 preparations, a
high percentage of the sperm with extruded fibers
showed a hook-like curve in the midpiece region (Table
1). These hook-like, post- disintegration forms bear a
marked resemblance to the hooked configuration of
Ca2+-treated sperm (Fig. 2).

TEM examination of the disintegrated sperm con-
ﬁrmed the reversed polarity of sliding produced by the
two methods. To identify individual microtubule-
ODFs, the numbering system first applied to mam-

Reagents
Ultrapure ATP and DTT used in reactivations were from
Boehringer Mannheim (Indianapolis, IN). Ultrapure CaCl2
and MgCl2 were obtained from Aldrich Chemical Co. (Milwaukee, WI). Electron microscopy reagents were ob-
tained from Polysciences, Inc. (Warrington, PA).
Fig. 1. Dark-field micrographs of rat sperm after disintegration by microtubular sliding. (A) Sperm models treated by incubation at low temperature and high pH (9.0) (LT/pH9 method) and disintegrated with 1 mM MgATP. Two isolated sperm are shown for detail, and a clump of several sperm is provided to show uniformity of the response. Note that the extruded bundle of fibers is from the side of the axoneme opposite the tip of the sperm head, and the structures remaining in the flagellar sheath do not exhibit any uniform pattern of curvature. (B) Sperm models treated by incubation at 37°C for 3 hours following reactivation with 0.3 mM MgATP and activation of motility with 3 μM cAMP (HT/pH7.8 method). Two isolated sperm are shown for detail and a clump of several sperm is provided to show uniformity of response. Note that the extruded bundle of fibers is from the side of the flagellum towards which the head curves (opposite to the configuration exhibited in A). The flagella of these sperm exhibit a pronounced hook-like curvature in the midpiece region, which is not seen in A, and these hooks strongly resemble the Ca²⁺-induced hooks of intact rat sperm models (see Fig. 2 for direct comparison). (C) Sperm produced by the same method as in B, but in this case after a prolonged incubation (~18 hours) at 23°C, after initial sliding was induced at 37°C. The sliding pattern and hooks seen in B are still present; however, additional fibers have emerged from the midpiece-principal piece junction. These additional fibers appear to originate from the side of the flagellum opposite that of the first extruded bundle, confirming that both sides of the axoneme are functional after the HT/pH7.8 method. Bar, 50 μm (for all three panels).
Table 1. The prevalence of strongly curved flagellar midpieces (hooks) in rat sperm disintegration

<table>
<thead>
<tr>
<th>Preparation method</th>
<th>No. of experiments</th>
<th>Total no. of cells counted</th>
<th>% of cells with hooked midpieces*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT/pH7.8 method</td>
<td>4</td>
<td>761</td>
<td>86.5%</td>
</tr>
<tr>
<td>LT/pH9 method</td>
<td>4</td>
<td>1412</td>
<td>3.6%</td>
</tr>
</tbody>
</table>

*Disintegrated sperm models with visible extruded fibers were counted as hooked if the midpiece (first 50 μm) of the flagellum exhibited a unidirectional curvature > 0.007 radians/μm.

Table 2. Axonemal elements observed in isolation

<table>
<thead>
<tr>
<th>Preparation method</th>
<th>No of hooked midpieces</th>
<th>Intact groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT/pH9+EGTA</td>
<td>140</td>
<td>53, 9, 34, 35</td>
</tr>
<tr>
<td>LT/pH9+Ca²⁺</td>
<td>85</td>
<td>33, 34</td>
</tr>
<tr>
<td>HT/pH7.8</td>
<td>14</td>
<td>22*</td>
</tr>
</tbody>
</table>

During the course of data collection for Fig. 5, a running count of isolated elements (appearing separated from partial or intact axonemes) was kept. The table shows the result of that running tally. Singly appearing 2,4,7 and 9 fibers were not counted, as they were too difficult to identify positively. Note that there is a rather direct correspondence in the numbers of isolated 3-central-8 complexes and 9-1-2 groups in the LT/pH9 method, suggesting that these are produced by fracture of residual axonemes into two parts. Intact 9-1-2 complexes are seldom found in the HT/pH7.8 preparations, but isolated 1 fibers are plentiful.

Fig. 2. Rat sperm flagellar response to Ca²⁺. These dark-field micrographs are of rat sperm extracted with 0.1% Triton X-100 in a glutamate-Tris buffer containing 0.5 mM EGTA. The hook-like curvature in the midpiece region is induced by addition of 1 mM CaCl₂ (for details of this response, see Lindemann and Goltz, 1988). These micrographs are provided for comparison with the flagellar configurations induced during sliding disintegration (see Fig. 1). The location and magnitude of the curve and overall shape of the flagellum are remarkably similar in sperm models treated either with Ca²⁺ or by the HT/pH7.8 disintegration protocol. No such similarity exists in the models treated by the LT/pH9 method of inducing disintegrations. Bar, 50 μm.

Table 1. The prevalence of strongly curved flagellar midpieces (hooks) in rat sperm disintegration

Rat sperm by Wooley (1974) was used (see Fig. 9 for reference). The 5-6 doublet-ODFs were most frequently absent in cross-sections of sperm prepared by the LT/pH9 method (Fig. 3). Inversely, the 9-1-2 group of doublet-ODFs were most consistently missing in cross-sections from HT/pH7.8 disintegrations (Fig. 4). Certain axonemal elements persistently remained associated even after disintegration of the axoneme.

In the LT/pH9 preparation, the principal piece cross-sections seldom lacked the 9, 1 or 2 fibers, but midpiece sections often had the 9-1-2 doublet group physically separated from the structure formed by the 3, central pair and 8 doublets (Fig. 3). These 3-central-8 structures could be seen in high numbers in relative isolation (Table 2). Likewise, a significant number of the 5-6 doublet-ODF combinations remained together after extrusion (Table 2).

In the HT/pH7.8 method, the mitochondrial remnant remains, but occasionally the axonemal remnant would split, with the 3-central-8 group separate from the 4-5-6-7 fibers (Fig. 4B). Sometimes, the extruded 9-1-2 doublet-ODFs could be observed sliding as a group (Fig. 4A), indicating that these elements occasionally remain associated during fiber extrusion. However, after extrusion, they seldom remained as an intact structure (<3%) (Table 2). In some principal piece sections, roughly the opposite pattern of fibers remained (similar to those left in the LT/pH9 group). Occasionally, a principal piece section was observed containing only the 3-central-8 complex (Fig. 4F, G, H). These are undoubtedly a consequence of disintegrations where fibers from both sides have slid (Fig. 4C). The 9-1-2 side slide from the neck region, and the 4-5-6-7 side slide from the midpiece-principal piece junction.

Sixty individual cross-sections from each of three preparations were analyzed for the presence or absence of each of the nine doublets. The results are displayed in Fig. 5. In Fig. 5A and B, the LT/pH9 method was employed with and without 1 mM added Ca²⁺. Individual doublet sliding was not altered. Fig. 5C displays the pattern of doublet-ODF sliding from an HT/pH7.8 preparation. Notably, while the pattern is reversed, the immobility of the 3-central-8 complex is conserved in both methods. The 3-central-8 complex is a stable structure, and does not participate in sliding of either polarity. The strong association of the 5-6 doublets is also maintained independently of the axoneme (Fig. 3 and Table 2). This association has been reported earlier in flagella (Olson and Linck, 1977; Linck, 1979) and cilia (Gibbons, 1961; Gibbons and Frank, 1972) and is confirmed by our results. The 5-6 doublets appear structurally fused or bridged and do not slide on one another. The axis of the 3-central-8 partition is parallel to the axis of the 5-6 complex. These structures impart an increased stiffness in the plane perpendicular to the plane of beating.

Flagellar curvature and fiber activation

Fig. 1 and Table 1 give qualitative and quantitative...
Fig. 3. Rat sperm disintegrated by the low temperature/pH 9 protocol (LT/pH9) as seen by transmission electron microscopy. (A-C) Sperm cross-sections in the principal piece region of the sperm. The loss of doublets and ODFs from the side of the axoneme made up of the 4,5,6 and 7 group is apparent (see Fig. 9 for the standardized numbering system). This is consistent with the dark-field observations of sliding polarity shown in Fig. 1A. The same pattern is also observed in the midpiece sections seen in A, D and F. However, because this protocol removes most, if not all, of the mitochondrial sheath, the midpiece sections are unbound and appear fragile. While most remain as a half axoneme, including doublet-ODFs 8,9,1,2,3 and the central pair, some axonemes fracture further into two distinct and separate subgroups. When this occurs, the two groups consist of the 9,1 and 2 doublet-ODFs as one structure and the 3, central pair and 8 as the other; D-G show this particularly well. The I-beam like central partition, formed by the 3 and 8 doublet-ODFs and central pair, is indicated by long arrows. In H, the central partition is so far removed from the rest of its axonemal elements that it appears to be in total isolation. Of the extruded group of fibers (4-7), only 5 and 6 were consistently found in association with each other after extrusion (short arrows). The micrographs shown are from an experiment in which 1 mM CaCl was present during disintegration; however, identical results were obtained in the absence of Ca²⁺ (see Fig. 5 and Table 2). x41,800.
Fig. 4. Rat sperm disintegrated by the high temperature/pH 7.8 (HT/pH7.8) protocol as seen by transmission electron microscopy. (A and B) Several flagellar cross-sections from the midpiece region of the sperm. The fibers that are missing or sliding are most often from the 9-1-2 group. In two of the cross-sections in A, this group is still present, but the ODFs are greatly reduced in size (arrows), indicating that the fibers have been partially extruded by sliding. It appears that the 9-1-2 group is sliding out as a unit. Unlike the LT/pH9 method, a mitochondrial remnant is still present surrounding the axonemes. Despite this mechanical limitation, the elements remaining in the axoneme are occasionally seen to separate into two subgroups as seen in B. One of these subgroups is composed of doublet-ODF complexes 8 and 3 in association with the central pair of doublets. This 3-central-8 partition is identical to those shown in Fig. 3, produced by the LT/pH9 protocol. (C, D and E) The pattern of residual fibers seen in principal piece cross-sections. The 9-1-2 set of doublet-ODFs is the predominantly missing group. Occasionally, principal piece cross-sections can be found that lack both the 9-1-2 and 4,5,6,7 doublet-ODFs. Three such cross-sections are displayed in F,G and H. These are most likely the result of the pattern of sliding seen in Fig. 1C, where fibers from both sides of the flagellum are evacuated. A central partition, formed by the 3-central-8 partition attached to the lateral columns of the fibrous sheath, remains intact in each of these cross-sections. x39,300.
The effect of pH 9.0 on curvature of rat sperm hooks

Fig. 7. The effect of pH 9.0 extraction on Ca\(^{2+}\)-induced hooks and sliding-induced hooks in rat sperm models. The results of three experiments are shown. In each experiment, the curvatures of the midpieces (first 50 μm) of at least 20 cells were averaged and are displayed before and after a 30 minute incubation at pH 9.0. Each graph displays the curvatures of sperm induced into hooks by Ca\(^{2+}\) addition (by the method of Lindemann and Goltz, 1988), compared with curvatures of sperm from the same rat in hooks elicited by the HT/pH7.8 disintegration method (slid). In each case, there is a marked decrease in curvature following incubation at high pH. In A and B, virtually no hooks remain after extraction at pH 9.0. In C, the hooks are reduced but not eliminated. We have arbitrarily assigned a positive curvature to the direction indicated by the curve of the sperm head; hence, hooks are negatively curved. High pH incubation was carried out at 23°C.

The effect of vanadate on curvature of rat sperm hooks

Fig. 8. The effect of 10 μM sodium vanadate on Ca\(^{2+}\)-induced hooks and sliding-induced hooks in rat sperm models. The results of three separate experiments are shown in A, B and C. In each experiment the average curvature (n > 20) of the flagellar midpieces (first 50 μm) is displayed before and after a 10 minute incubation with 10 μM sodium vanadate. Each graph shows the curvature results of sperm with hooks induced by Ca\(^{2+}\) addition immediately after reactivation (by the method of Lindemann and Goltz, 1988), versus sperm (from the same rat) in hooks produced by the HT/pH7.8 protocol (slid). Incubation in vanadate causes a marked decrease in the negative curvature of hooks generated by either method. A negative curvature corresponds to a curve in the opposite direction to the curve of the sperm head. Vanadate incubation was performed at 23°C.

Discussion

Our results suggest that the mammalian sperm axoneme is segregated into three functional groups: (1) a medial 3-central-8 partition formed by the central pair linked to outer doublets 3 and 8 (in the midpiece, the 3 and 8 doublets are attached to ODFs 3 and 8 and in the principal piece to the fibrous sheath); (2) the doublets 9,1 and 2 that slide as a group through the interaction of doublet 2 with doublet 3 of the central partition; (3) the 4,5,6,7 doublet group (in which 5 and 6 are permanently linked) that also appears to slide as a group on the center partition.

The 3-central-8 partitions seen in the midpiece region, in both the HT/pH7.8 and LT/pH9 methods, are suggestive of an I-beam. An I-beam is widely employed in architecture to provide maximal strength in one direction (usually the direction bearing weight) with a minimal expense of material. Here, nature may have employed the I-beam construct to provide a maximal stiffness differential in one plane of the flagellum relative to the orthogonal plane. Olson (1979) and Oko (1988) showed that the fibrous sheath of rat sperm has a partition-like component that crosses the axoneme in the plane of the central pair. This reinforcing structure
Fig. 9. A schematic representation of the functional organization of the rat sperm axoneme. A tracing of an electron micrograph is shown with each doublet-ODF pair labeled using the numbering system adopted from Wooley (1977). The axoneme has been subdivided, based on our morphological and functional results. The 3-central pair-8 complex forms an I-beam-like partition dividing the axoneme into three functional groups. The 5-6 elements are permanently bridged to form a large, ribbon-like structure located opposite to the large, flattened number 1 ODF. In the upper drawing, the 4,5-6,7 group is shown outside the sheath, as it appears after the LT/pH9 disintegration method. In the lower drawing, the 9-1-2 group is shown emerging from the midpiece-head junction, as it does in the HT/pH7.8 method of inducing disintegration.

may explain the stability of the 3-central-8 partition in the principal piece (Fig. 4F,G,H).

Our work also confirms the earlier observation that the 5-6 tubules are solidly associated in mammalian sperm (Olson and Linck, 1977; Linck, 1979). This connection makes their structure more similar to a flat ribbon than to two pillars. The finding that the 9-1-2 tubule-ODFs often slide as a group implies that they may also act more like a ribbon than like three independent elements, and ODF number 1 is flattened to maximize its stiffness in the plane perpendicular to the beat.

The functional organization suggested by our results is summarized in Fig. 9. This view is consistent with Fawcett’s earlier suggestions (1975) for the way the mammalian sperm axoneme might function. It explains the planar beating of the axoneme and limits sliding to only certain doublets during each phase of the beat cycle.

Whether our findings are applicable to cilia and flagella in general, or unique to modified flagellar structures, is yet unclear. Tamm and Tamm (1984) reported very similar results in the large compound cilium of the ctenophore Beroe. In that system, the 3-central-8 complexes of many aligned axonemes are linked to produce a large coordinated multiaxonemal structure that beats as a unit. Other investigators attained other axonemal dissociation patterns using simple 9 + 2 cilia and flagella. Sale (1986) reported results similar to our HT/pH7.8 result, while Satir and Matsuoka (1989) found patterns of sliding showing no evidence of a stable 3-central-8 partition. Furthermore, Shinyoji et al. (1991) recently reported that the plane of beating is not mechanically determined in sea urchin sperm, but may be altered by initiating bending in a new plane using external vibrations. This suggests that the particular doublets activated are more important than the mechanical constraints of the flagellum in determining the beat plane.

Possibly, in simple flagella, mechanical restrictions determine the beat plane by selecting the doublet pairs initially activated in each cycle. This is the only interpretation consistent with most observations that could apply equally to sea urchin and rat sperm. However, our results, and those of Tamm and Tamm (1984), suggest that the 3-central-8 partition may serve as an adaptation allowing construction of large mammalian sperm flagella and compound cilia from the basic axonemal structure.

In the HT/pH7.8 protocol, the 9-1-2 group usually slides in a headward direction. The dynein arms between doublets 2 and 3 are responsible for the expulsion of the 9-1-2 bundle. These arms appear to be deactivated by high pH extraction, on the basis of the fact that sliding of the 9-1-2 bundle does not occur in the LT/pH9 method, but occurs preferentially in the HT/pH7.8 method. In addition to the 2-3 interdoublet dynein arms, the 3-4 and 4-5 interdoublet dynein arms also bend the flagellum in the direction which forms the Ca$^{2+}$-induced hook. The structure remaining after the 9-1-2 group is extruded usually maintains a hook-like bend in the midpiece. These hooks resemble the Ca$^{2+}$-induced hooks of intact sperm models in both shape and bend polarity.

Whether the midpiece hooks observed following HT/pH7.8 axonemal disintegration of swimming models represent the same phenomenon as the Ca$^{2+}$-induced hooks (Lindemann and Goltz, 1988), or is coincidentally similar artifact, cannot definitely be determined at this time. Using the HT/pH7.8 method, the outer dense fibers exhibit a strong circular curvature of their own after extrusion. The remaining ODFs may impart the midpiece hook because their own curvature is now unopposed, due to the lack of fibers on the opposite side (see Fig. 1B). However, on the basis of our observations with vanadate, it is more likely that the mechanisms producing the hooks in the disintegrated sperm and producing the Ca$^{2+}$-induced hooks
are the same and both depend on the action of dynein arms. The unopposed action of the 3-4 and 5-6 interdoublet dynein arms (contributing to bending in the same direction as the 2-3) is the most likely origin of the Ca\(^{2+}\)-induced hooks, and Ca\(^{2+}\) may activate these arms selectively in the fully intact axoneme. This last concept would be consistent with the Ca\(^{2+}\)-regulated switching mechanism proposed by Satir (1985).

It has previously been suggested that separately activated subpopulations of dynein bridges may contribute to function in the mammalian sperm. Rosenthal and Linck (1979) reported that sliding of element no. 4 of the rat sperm axoneme was influenced by pH, and Mohri and Yano (1982) reported light-microscopic evidence that the disintegration pattern of hamster sperm flagella was altered by the presence or absence of calcium. These preliminary studies clearly implicated both Ca\(^{2+}\) and pH as having selective activating/de-activating effects on particular dynein arms. Our results substantiate the concept of two separately activated populations of dynein arms in the axoneme. Using high pH extraction, the 9-1-2 microtubule-fiber group was either never extruded, or extruded so rarely that it was undetectable in EM examination of the cross-sections. This is identical to the earlier result obtained by Olson and Linck (1977) using the same method. The mitochondrial sheath is eliminated in these sperm, leaving no apparent mechanical impediment to sliding at the 2-3 interdoublet dynein arms. Therefore, we conclude that the 2-3 sites are inactive after extraction at pH 9.0. In contrast, the extrusion of the 9-1-2 bundle in the HT/pH7.8 method can only occur by sliding at the 2-3 interdoublet dynein arms. As already noted, the midpiece hooks, seen after sliding is induced by the HT/pH7.8 method, most likely result from tension produced at the 3-4 and 4-5 arms. Therefore, it is likely that in the HT/pH7.8 method, these dynein arms are also active. This interpretation is further supported by our experiments with vanadate and pH. It is interesting to note that, in the method employed by Rosenthal and Linck (1979), only one side of the axoneme (4,5-6 and 7 doublet-ODFs) participated in sliding. This would indicate that only element 4 of the axonemal elements 1,2,3,4 and 5 (which work together in one bend polarity) was effected by pH. This is the same side we found to be pH sensitive.

Ca\(^{2+}\)-induced hooks, and hooks formed after sliding by the HT/pH7.8 method, were both reduced or eliminated following high pH (9.0) extraction. Vanadate at 10 μM also partially removed either form of hook. This suggests that, in both cases, the hooks are derived from a sliding and bridging at the 2-3, 3-4 and 4-5 dynein arms. Sale (1986) reached a similar conclusion from disintegration of sea urchin sperm flagella. However, he determined that the opposite microtubule pairs, 6-7, 7-8 and 8-9, were Ca\(^{2+}\) activated. He made this deduction because the 4 through 7 doublets slid baseward with Ca\(^{2+}\) present. This is roughly the equivalent of the high pH (LT/pH9) result. Fortunately, the asymmetric rat sperm head gives us a structural marker for evaluating the direction of bending. We know that the Ca\(^{2+}\)-induced direction of bending must activate the 2-3, 3-4 and 4-5 dynein arms if the interdoublet sliding is producing the bending. The polarity of the rat sperm axoneme (with respect to the head) has also been ascertained independently by Wooley (1977). Our determination of polarity is consistent with his. Therefore, the Ca\(^{2+}\)-dependent bend in sea urchin and rat sperm must be produced by opposite sets of dynein arms, or the bend that Sale (1986) assumed to be in the Ca\(^{2+}\)-hooked direction was morphologically similar, but in the opposite bending direction. In mammals, the arms that impart Ca\(^{2+}\) sensitivity may be the opposite to those in invertebrates. The direction of Ca\(^{2+}\)-induced bending between rat sperm and mussel gill lateral cilia was also reversed in relation to tubule numbering (Satir et al., 1992).

Two unambiguous deductions can be made from our results: (1) high pH inactivates the dynein arms for one pattern of disintegration but not the other; (2) the arms inactivated by high pH are required for the Ca\(^{2+}\)-curvature response and for flagellar bending in that direction. A third finding is also obvious, but puzzling: while these arms seem to play a role in the Ca\(^{2+}\) response, they can still actively slide even in the absence of Ca\(^{2+}\). There are two possible explanations: Ca\(^{2+}\) may bias these arms, making activation difficult when Ca\(^{2+}\) is absent and progressively easier when the Ca\(^{2+}\) level increases. Alternately, calcium may lock the arms into a rigor-like state at the end-point of the beat cycle. The first explanation is the most plausible, since the response to Ca\(^{2+}\) often occurs gradually (over many beat cycles) and can proceed in low levels of vanadate (< 2 μM), where there is no flagellar beating (Linde mann and Goltz, 1988).

In conclusion, there appears to be a stable partition bisecting the rat sperm axoneme. This partition is composed of the 3 and 8 doublets connected through the central pair by the spoke apparatus and includes the 3 and 8 ODFs in the flagellar midpiece, or is connected to the lateral columns of the fibrous sheath in the principal piece. Flagellar bending occurs through the sliding of the 9-1-2 and 4,5,6,7 doublet-ODF groups upon this stable central partition. The 9-1-2 group is translocated base-ward by the action of the 2 to 3 interdoublet dynein arms, and the 5-6 complex is moved base-ward by the 6 to 7 and 7 to 8 interdoublet dynein arms. Functionally, the rat sperm axoneme does not act like 9 independent, circularly arranged elements, but instead resembles a series of flattened ribbons sliding on an I-beam-like central partition. Interdoublet sliding in the two bending directions is differentially sensitive to extraction at pH 9.0. Following extraction at high pH, the Ca\(^{2+}\) sensitivity of the axoneme is lost, coincident with the loss of function at the 2-3 dynein arms. The existence of two distinct populations of dynein bridges seems certain, and the flagellar Ca\(^{2+}\) response appears to be associated with one set (microtubules 2-3, 3-4 and 4-5).

The authors thank Mrs. Loan Dang and Mrs. Mei Cheng...
for help with thin-sectioning and TEM techniques. This work was supported by NSF grant no. DCB-8819034.

References


(Received 14 October 1991 - Accepted, in revised form, 27 February 1992)