Interdoublet Sliding in Bovine Spermatozoa: Its Relationship to Flagellar Motility and the Action of Inhibitory Agents

ZACHARY BIRD
Department of Biological Sciences, Oakland University, Rochester, Michigan 48309

ROBERT HARD
Department of Anatomy and Cell Biology, SUNY at Buffalo, Buffalo, New York 14214

AND
KATHLEEN S. KANOUS AND CHARLES B. LINDEMANN
Department of Biological Sciences, Oakland University, Rochester, Michigan 48309

Received November 22, 1995, and in revised form March 11, 1996

Interdoublet sliding rates were assessed in bull sperm, utilizing a freeze–thaw procedure to allow axonemal disintegration. The sliding rate at 23°C increased with increasing MgATP concentrations up to 1 mM ATP, to plateau at 8 μm/sec. The analyzed interdoublet shear in both live and demembranated (Triton X-100-extracted) bull sperm reactivated with 1 mM ATP established maximal microtubule sliding rates at 6 μm/sec during flagellar beating. Therefore, in vitro sliding rates were sufficient to account for the beat in intact flagella. The effect of inhibitors of flagellar motility on in vitro sliding rates was evaluated. While 8 μM vanadate minimally reduced the sliding rate (to ~ 4 μm/sec), only 0.5 μM vanadate was sufficient to terminate reactivated bull sperm motility. Nickel ion (0.66 mM) terminated all spontaneous motilility, while only reducing microtubule sliding rates to ~ 5.0 μm/sec. Exposing intact bull sperm to theophylline (1 mM), and incubating the subsequently demembranated sperm in cAMP (3 μM), improved flagellar motility, but had little impact on microtubule sliding rates as determined by axonemal disintegration. Furthermore, deactivating live sperm with 2 mM KCN and 4 mM 2-deoxy-D-glucose renders the subsequently reactivated sperm immotile (as long as exogenous cAMP is absent). Yet, this treatment only reduced the sliding rate by 38%. Paradoxically, 4 mM MgADP reduced the sliding rates most dramatically (86%), whereas demembranated sperm models retain a strong, coordinated beating pattern in the presence of MgADP. These results demonstrate that there is no direct relationship between interdoublet sliding rates and the capacity for coordinated flagellar beating. © 1996 Academic Press, Inc.

INTRODUCTION
The motion of flagella is driven by force produced through the interaction of tubulin and the motor protein dynein. A dynein arm located on one outer doublet attaches to the adjacent doublet and undergoes a power stroke driven by the hydrolysis of ATP. The interdoublet force produced causes a sliding displacement between those two outer doublets (Satir, 1968). In an intact axoneme, structural connections limit the sliding displacement, resulting in a flagellar bend, thus creating a beat. Structurally modifying the axoneme by digesting the nexin and spoke connections (or rupturing these structures) allows the doublet microtubules to slide continuously (Summers and Gibbons, 1973; Lindemann and Gibbons, 1975). Sliding is observed between adjacent doublet microtubules, with the exception that in some cilia and flagella doublets 5 and 6 are permanently bridged (Afzelius, 1959). It has also been demonstrated that each microtubular doublet slides base-ward on its higher numbered neighbor (Sale and Satir, 1977).

The primary goal of this study was to directly examine dynein–tubulin sliding in a mammalian sperm. Microtubule sliding rates would be assessed, and the relationship between individual doublet sliding rates and the rates needed to propel normal bending/beat in flagella would be determined.

A simple method developed earlier (Lindemann et
was used to allow flagellar axonemes to slide apart. This technique relies on the action of freezing the cells at ~20°C with Triton X-100 and dithiothreitol (DTT) to remove the mitochondrial sheath. Using this method, the relationship between interdoublet sliding rates and Mg-ATP concentration was examined.

Additionally, several compounds previously identified as either stimulatory or inhibitory to flagellar motility (KCN, VO$_4^{2-}$, Ni$^{2+}$, and ADP) were examined for their effects on sliding. Each of these agents has demonstrated specific effects on flagellar motility. Adding cAMP to demembranated cells demonstrably increases flagellar beat frequency. In fact, completely immotile cells will spontaneously regain lost motility following cAMP addition (Lindemann, 1978). Exposure to KCN and 2-deoxy-d-glucose (2-DG) (which in combination compromise a step in the kinase-A pathway) greatly reduces the phosphorylation state of intact sperm and blocks motility and energy metabolism. Furthermore, the effects can be reversed by adding cAMP (an integral constituent of the kinase-A pathway). Adding theophylline to live cells increases cAMP by inhibiting phosphodiesterase, the enzyme that normally breaks down cAMP.

Sodium vanadate can completely inhibit flagellar motility. The vanadate ion binds directly to the flagellar dynein, prohibiting the dynein arms from attaching to and forming cross-bridge attachments with tubulin (Sale and Gibbons, 1979). NiSO$_4$ treatment prevents the formation of flagellar waves, impeding subsequent swimming motility. However, manually introducing a bend onto the flagellum reinitiates wave propagation and microtubule sliding (Lindemann et al., 1980). ADP affects flagellar beat frequency by interfering with the mechanochemical cycle of dynein. Movement is altered when ADP competes for the phosphate site that normally binds ATP (Okuno and Brokaw, 1979).

The present study tested these agents for their impact on microtubule sliding rates. The effect each had on the pattern of axonemal disintegration was also noted. The interdoublet sliding rates in both live, intact sperm and motile, detergent-extracted sperm models were also analyzed as a comparison to the interdoublet sliding rates obtained from disintegrating axonemes.

**MATERIALS AND METHODS**

**Sperm Preparation**

Bovine spermatozoa (~7.5 × 10$^6$ per milliliter), suspended in tris(hydroxymethyl) aminomethane (tris)/sodium citrate extender containing 28% egg yolk (at a pH between 6.5 and 7.0) was shipped from NOBA, Inc. (Tiffin, OH). These cells were transported in a container with ice, received in the laboratory the next morning, and subsequently stored at 0–5°C for the brief period before experimental procedures were carried out. The egg yolk extender was removed by washing 2 ml of the sperm cell suspension with citrate buffer (0.097 M sodium citrate, 5 mM MgSO$_4$, and 2 mM fructose at a pH between 7.3 and 7.4). The washing involved centrifuging the mixture at 960g for 10 min, followed by decantation, resulting in a sperm cell pellet. The pellet was resuspended in the citrate buffer and recentrifuged. The supernatant was again removed, and enough citrate buffer was added to the final pellet to bring the volume of the washed sperm suspension back up to the original 2 ml volume.

**Mitochondrial Stripping and Microtubule Sliding**

For each microtubule sliding rate experiment, 100 μl of the sperm suspension was added to a petri dish containing 3 ml of a demembranating mixture (0.024 M potassium glutamate, 0.13 M sucrose, 0.002 M Tris–HCl, 0.5 mM EGTA, 1 mM MgCl$_2$, 1 mM DTT, and 0.1% Triton X-100). The dish was then frozen at ~20°C for 48–72 hr, a procedure that stripped the mitochondria from the midpiece region of the flagellum.

A perfusion chamber was constructed of a glass slide or long, glass coverslip, with the coverslip placed in a thin line along each of the long edges, onto which a long glass coverslip was seated. This produced a shallow perfusion chamber between the slide/cover slip and upper coverslip. The thawed cell suspension was injected into the perfusion chamber using a micropipette, and cells were allowed to sink to the bottom of the chamber. When the flat side of the bovine spermatozoan heads contacted the flat glass surface of the chamber bottom, some of the sperm heads became attached to the perfusion chamber. Superfusates (those not adhered to the chamber) were removed by adding demembranating media at one end of the chamber and drawing the fluid through the chamber from the opposite edge using filter paper. This perfusion process also caused the flagella of the fixed cells to point in the same direction.

Subsequently perfusing additional demembranating media, containing 1 mM MgATP, induced extrusion of the outer microtubules and associated outer dense fibers (ODFs) from the flagellar midpiece. The sliding rate experiments using Ni$^{2+}$ and VO$_4^{2-}$ included an additional step. The specific inhibitor (included in the demembranating mixture) was perfused, followed by an incubation period of approximately 10 min, prior to perfusing more of the same solution containing 1 mM MgATP. The protocol of the ADP experiments was slightly different. The incubation period was decreased to 30 sec, and Mg$^{2+}$ concentrations in the media were not fixed, but were maintained equimolar to the nucleotide (ADP or ATP) concentrations. The nickel experiment protocol also differed, omitting DTT, after the stripping protocol because the combination of Ni$^{2+}$ and DTT often forms a precipitate.

In the KCN/theophylline version of the sliding rate experiments, the sperm were pretreated while still alive (prior to demembranation with Triton X-100). Each experiment included both a theophylline and a KCN sample from the same original sperm stock sample, though each involved a different setup procedure. For the theophylline sample, cells were washed with citrate buffer and brought to a final volume of 5 ml. Theophylline (1 mM) was then added. The motility state of the cells was analyzed by adding 100 μl of these cells to a dish containing reactivation media (demembranation media plus 1 mM MgATP). If vigorous swimming was exhibited by these sperm “models” (demembranated cells reactivated by the addition of MgATP), the theophylline treated sample was considered acceptable. Another 100-μl fraction of the intact theophylline-treated sperm sample was then subjected to the mitochondrial stripping protocol outlined above.

Cells for the KCN sample were washed in citrate buffer without fructose, bringing the final volume to 5 ml. KCN (2 mM) and 2-DG (4 mM) were added to these intact cells. The samples remained at room temperature for an additional 1 to 1.5 hr. and were covered to decrease the O$_2$ levels. The cells were checked periodically for motility, and when a cessation of movement was
observed, 100 µl of these cells was added to reactivation media and examined for motility. If the cells demonstrated an absence of swimming (little or no movement), the KCN sample was considered to be sufficiently inhibited. A separate 100-µl aliquot of the intact, KCN-treated cell suspension was then subjected to the freezing/striping protocol, with 19,500 units of protein kinase inhibitor (PKI) added to the dish to prevent the possibility of endogenous kinase-A rephosphorylating the axoneme. Following thawing, microtubule sliding rates were assessed using 1 mM ATP for the KCN sample and 1 mM ATP with 0.003 mM cAMP for the theophylline sample.

Microtubule sliding rates were calculated by analyzing the videotape recordings of each experiment. Individual sliding rates were found by measuring the length of an extruded microtubule bundle (measuring the loop formation at a single frame), subtracting the length of the residual (unslid) portion of the axoneme, and dividing that length by the time required to form the loop (µm/sec). Measurements were taken while each bundle was still intact (not splayed into individual microtubules/ODFs). A possible variance in sliding rates over time was compensated for by measuring rates of both small (early) and large (late) extruded loops.

For some of the experiments, interdoublet sliding was imaged using high extinction DIC optics (objective magnification = 40×, N.A. = 0.65) on a Zeiss UEM microscope (Carl Zeiss Inc., Thornwood, NY). The images were captured with a Dage MTI Newvicon video camera (Model NC 67M, Dage MTI, Inc., Michigan City, IN) and analogue processed with camera gain and black level controls. The camera output was passed through an Argus 10 Image Processor (Hamamatsu Photonics, Inc., Oakbrook, IL) for on-line background subtraction. High resolution super-VHS videotape recording were made using a JVC HR-S10000U SVHS video recorder (JVC Co. of America) to document the disintegration events. On one occasion, the output of the Argus 10 was digitized as a series of movies at 1-sec intervals with a Scion Frame Grabber (Model VI 1200, Scion Corp., Frederick, MD) on a Macintosh IIx microcomputer using the public domain NIH Image program (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov). The images were stored on disk and selected frames were printed on Kodak Technical Pan 35-mm negative film (Eastman Kodak Co., Rochester, NY) using a Lasergraphics slide maker and Aldus Persuasion software (Aldus Corp., Seattle, WA).

The flagellar beating motion of live sperm cells, modeled cells in ATP reactivation media, and reactivated cells in the presence of inhibitors (83 nM VO₄⁻, 0.66 mM Ni²⁺, or 2 mM ADP) were observed. The attachment of the flat bovine sperm head to the culture dish or perfusion chamber allowed the observation of flagellar beating with the sperm head stationary. The flagellar beat was videotaped using strobe lighting, resulting in clear single frame observations, enabling the construction of individual, timed tracings. A full beat cycle was traced for each cell analyzed. Shear angles, measured at 5-µm intervals along the flagellum, were calculated and plotted versus the distance from the sperm head.

The product of the shear angle and the interdoublet spacing in the bending plane is a fairly direct estimate of sliding displacement in flagella (Brokaw, 1989). In mammalian sperm, the true interdoublet spacing is complicated by the attachment of the doublets to the outer dense fibers along much of the flagellar length. The shear angle measurements were used to estimate sliding between the doublets of the bull sperm axoneme over the course of the beat cycle. To correct for the fact that the doublets of a bull sperm are anchored to the connecting piece through the ODFs, the center to center distances between the ODFs were used to find the sliding contributed by each 5-µm segment. The sum of these values constituted the cumulative sliding along the flagellar length. Rates were found by taking the difference in total sliding between each subsequent tracing. All of the necessary calculations were accomplished with the aid of a QuickBASIC computer program written for the purpose of aiding the data analysis.

RESULTS

Transmission electron microscopic (TEM) evidence was used to determine specifically which microtubules were being extruded from the axoneme (Figs. 1 and 2). In control samples (microtubules un-
impeded by midpiece mitochondria and allowed to slide with no inhibitor present), electron micrographs ascertained that elements 4, 5–6, and 7 (utilizing the numbering system of Afzelius, 1959) are initially extruded out of the axoneme as a single group or bundle (see Fig. 1). From this evidence, it was surmised that doublet 7 slid out on doublet 8 to extrude the 4, 5–6, 7 bundle as a single loop. The videotaped activity of this sliding bundle was used to calculate the microtubule sliding rate (doublet 7 sliding on doublet 8). No variance was observed in sliding rate over time, as loop measurements were taken both early (small loops) and later (larger loops) in the sliding process. The TEM results were augmented by duplicating the experiments in the research facilities of Dr. Robert Hard at SUNY (Buffalo, NY), where sliding was observed utilizing Differential Interference Contrast (DIC) optics during videomicroscopy and video image enhancement (Fig. 3). Utilizing this equipment, it became possible to observe both the initial emergence of each microtubule bundle and the point at which each bundle splits into individual elements. The extruded loop usually divided upon reaching an average length of 37 ± 8 μm (n = 13). These results confirmed that the sliding rate data collected previously (by videomicroscopy) represent the rates of a single doublet (7) translocating on its adjacent neighbor (8). Since the total flagellar length is approximately 60 μm, this means that the emerging bundle has slid approximately halfway out of the sheath at the point that the bundle undergoes further fragmentation.

The effects of Mg-ATP on interdoublet microtubule sliding were assessed at seven different molar concentrations. A low concentration of ATP (0.021 mM) resulted in a sliding rate of 1.82 ± 0.23 μm/sec. Incremental increases of ATP produced proportional increases in the sliding rate. However, higher levels of ATP (1.0 and 2.0 mM) generated comparable microtubular sliding rates of 8.00 ± 1.96 and 8.08 ± 2.28 μm/sec respectively, indicating a plateau was reached at ATP levels in the range of 1.0–2.0 mM (Fig. 4). Sliding rates at ATP concentrations greater than 2.0 mM were not assessed.

The effects of sodium vanadate (NaVO₃) on microtubular sliding were evaluated at four different molar concentrations (Fig. 5). At 2 μM NaVO₃, sliding was measured at a rate of 5.76 ± 1.35 μm/sec. Increasing the vanadate concentration to 4 μM, reduced the sliding rate to 4.71 ± 1.52 μm/sec. At 8 μM NaVO₃, the rate decreased to approximately half that of the control (which slid at a rate of ≈8.0 μm/sec). It was also noted that the extruded microtubules of these cells often remained in a tight bundle and did not split into each separate element, even long after extrusion of the initial bundle (>10 min later). In addition, the microtubules forming these unbroken “vanadate loops” usually stopped sliding before the extruded bundle freed itself completely from the sheath. Comparative data showing the persistence of unbroken loops relative to increasing vanadate concentrations is presented in Table I. Vanadate concentrations of 8 μM or higher greatly reduced the number of disintegrating cells, while 8 μM vanadate only decreased the sliding rate to half of the uninhibited value (Table I). High levels of vanadate drastically reduced the number of disintegrating cells, making it difficult to obtain sliding

**Fig. 2.** TEM micrographs of nickel-inhibited, disintegrated bovine sperm axonemes. Nickel (at 0.66 mM) inhibits the sliding of doublets 9, 1, and 2, resulting in the extrusion of only the group of doublets/outer dense fibers on the opposite side of the axoneme (4 through 7). ×67 600.
rates at concentrations greater than 8 $\mu$M. However, diligently repeating the experiment at 16 $\mu$M vanadate gave a sliding rate of 2.13 ± 0.64 $\mu$m/sec from the limited number ($n = 6$) of cells that did undergo sliding.

Microtubular sliding rates were calculated for samples subjected to two different nickel sulfate concentrations (Fig. 6). At 0.66 mM Ni$^{2+}$, a rate of 5.36 ± 1.19 $\mu$m/sec was observed, while 1.33 mM nickel diminished the rate to 4.54 ± 1.09 $\mu$m/sec. These high levels of Ni$^{2+}$ only suppressed the sliding rate, without greatly reducing the percentage of disintegrating cells (data presented in Kanous et al., 1993). However, it is noteworthy that Ni$^{2+}$ altered the final pattern of microtubule sliding observed in TEM. Control cells expelled elements 4, 5–6, 7 and sometimes subsequently expelled elements 9, 1, 2 (see Fig. 1). Ni$^{2+}$-inhibited cells generally only extruded the 4, 5–6, 7 side of the axoneme, as demonstrated in Fig. 2.

The effect of MgADP on the microtubule sliding rate of bovine sperm in the presence of 1 mM ATP is demonstrated in Fig. 7. Adding ADP dramatically reduced the sliding rate (3.43 ± 0.95 at 2.5 mM and 1.10 ± 0.10 at 4 mM), in spite of the fact that the ATP and ADP concentrations employed are compatible with sustained, coordinated beating in Triton X-100-extracted sperm models. Mg$^{2+}$ concentration was elevated parallel to the ADP concentration to assure that the reduction in sliding rates was not attributable to restrictions of available MgATP.

cAMP kinase-A regulation was explored by pretreating live sperm with theophylline for 5 to 10 min prior to demembranation, and then subsequently adding cAMP to the demembranated cells before subjecting them to the freeze/thaw protocol. These cells were compared to KCN/2-DOG-inhibited intact cells which were further treated with PKI after demembranation and subsequently subjected to the freeze/thaw process. The effect of these pretreatments on the resultant sliding rates are illustrated in Fig. 8. The reduction in sliding rate observed in the KCN/2-DOG-treated samples was similar in
magnitude to that of nickel-inhibited samples (4.80 ± 1.66 μm/sec). As reported earlier (Lindemann, 1978), KCN/2-DOG-treated intact cells that were subsequently demembranated showed only poor motility with MgATP, while those treated with theophylline showed vigorous motility under the same conditions.

Sliding rates for intact, beating flagella were also calculated with the aid of the aforementioned Quick-BASIC program (Fig. 9). Motility analysis of live cells revealed sliding rates in a range between 0 and ≈6.5 μm/sec, depending upon the area of the flagellum under observation. Flagellar motility was also analyzed in sperm “models” (cells demembranated with Triton X-100 and reactivated with 1 mM ATP). Reactivated cells exhibited microtubule sliding rates ranging from 0 to 7.0 μm/sec (see Fig. 10).

**DISCUSSION**

Flagellar motility is powered by the sliding of adjacent microtubule doublets, caused by the interac-

**TABLE I**

Disintegration of the Bull Sperm Axoneme in the Presence of Vanadate

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total No. of cells observed</th>
<th>Percentage of cells disintegrated</th>
<th>Percentage of cells with loops</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>233</td>
<td>80%</td>
<td>0.2%</td>
</tr>
<tr>
<td>2 μM vanadate</td>
<td>195</td>
<td>69%</td>
<td>17%</td>
</tr>
<tr>
<td>4 μM vanadate</td>
<td>263</td>
<td>64%</td>
<td>29%</td>
</tr>
<tr>
<td>8 μM vanadate</td>
<td>285</td>
<td>38%</td>
<td>22%</td>
</tr>
<tr>
<td>16 μM vanadate</td>
<td>364</td>
<td>14%</td>
<td>9%</td>
</tr>
</tbody>
</table>

---

*a* All experiments were performed on frozen–thawed samples of Triton X-100-extracted sperm (see Materials and Methods). All were performed with 1 mM MgATP at 23°C.

*b* All percentages in the table are calculated from the numbers given in this column.

*c* Disintegrated cells include those displaying axonemal element extrusion, either complete removal from the sheath or “loops” (see below).

*d* “Loops” are formed when the axonemal elements are not completely extruded within 10 min of ATP addition. The end of the element remains within the sheath, giving the appearance of a loop.
tion of dynein (an ATPase) and tubulin, and driven by the hydrolysis of ATP. In this study, interdoublet sliding was directly observed in frozen/thawed bull sperm that disintegrated by sliding apart upon MgATP addition to the thawed sample. An initial splitting of the axoneme into two sections was observed. A residual fragment remained in the sheath, while a mobile bundle buckled out of the flagellum from the midpiece region. Videotaped recordings indicate that this mobile bundle emerged to form a single loop (average length, $37 \pm 8 \mu m$) prior to undergoing a secondary splitting. TEM of cross sections from the disintegrated samples confirmed that the first group of subfibers to break away from the axoneme consisted of elements 4 through 7. The most frequently observed splitting pattern was missing all four of these elements. If the sliding polarity observed in other cilia and flagella is correct for bull sperm, the emerging bundle was driven by doublet 7 sliding on doublet 8. A substantial subpopulation of the examined cross sections were only missing elements 4 through 6. In these axonemes, the initial split must have occurred between doublets 6 and 7. Consequently, the recorded sliding rates generally represent sliding between doublets 7 and 8, but also included some splits where sliding occurred between doublets 6 and 7.

Early studies of sperm microtubular sliding disintegration employed trypsin to partially digest the axoneme, thus permitting the doublets to slide apart (Summers and Gibbons, 1971; Lindemann and Gibbons, 1975). Exogenous proteases were not employed in this study, yet the ATP-induced axonemal disintegration, following 72-hr frozen incubation, was immediate and quite reproducible. Additionally, once the samples were perfused with the ATP-containing solution, any residual proteolytic enzymes should have been eliminated through the perfusion, thus terminating the possibility of further degradation. Even if some proteolytic activity was involved in the disintegration procedure, it should be fairly uniform under all test conditions since all were initially prepared using the same demembranation/freeze–thaw protocol.

**FIG. 7.** The effect of ADP on microtubule sliding rates in bovine sperm disintegration in the presence of 1 mM ATP. The addition of MgADP causes a dramatic decrease in sliding rate, even though utilizing comparable concentrations of ADP in reactivated sperm models does not suppress a coordinated flagellar beat. Mg$^{2+}$ levels were increased proportionately to the increased ADP levels to avoid any potential activity constraints due to limited MgATP availability.

**FIG. 8.** The effect of cAMP kinase-A regulation on bovine sperm disintegration induced by 1 mM ATP. Live sperm samples pretreated for 1 hr with 1 mM theophylline prior to demembranation (with 3 mM cAMP added to the reactivation) are compared to those pretreated with 2 mM KCN and 4 mM 2-DOG for 1 hr prior to demembranation (with 19 500 units of PKI added to the reactivation). The theophylline pretreated sperm models were vigorously motile in 1 mM ATP, while the KCN/2-DOG-treated models were immotile in 1 mM ATP. Microtubule sliding rates decrease when cells have been cAMP depleted with KCN/2-DOG exposure of the live cells prior to the freeze/thaw protocol.
inhibitor capable of blocking acrosin) without an observable impact on the resultant sliding. Therefore, while intuition would support the involvement of proteolysis in this procedure, there is no conclusive evidence for its participation. These considerations introduce the possibility that just the removal of the mitochondrial sheath may be sufficient to allow the axoneme to split under the strain generated by microtubule sliding. If substantiated, this may provide insight into the structural significance of the sheath in mammalian sperm. This concept certainly warrants further investigation.

It was recognized that the absolute sliding rates could conceivably have been influenced by undetected splitting of the initial bundle during the time interval used to measure loop lengthening. This error would augment the measured sliding rate, by the addition of the rate from the sliding between another doublet pair. Since observations were generally made using the most visible element of the loop (i.e., combined elements 5 and 6), this would allow some data to be based on the summed rate of two doublets sliding simultaneously (the 7 sliding on the 8, and the 6 sliding on the 7). This potential source of error might have resulted in a fractional increase in the measured rate (with the upper limit of error being a factor of 2). However, based on the DIC analysis, when sliding rates were obtained from microtubule loops less than 36 μm in length, the error was negligible. MgATP delivery to the extracted cells generated another potential source of error. The mixing of media at the perfusion boundary, plus the presence of the fibrous sheath, would tend to delay direct application of the full ATP concentration to the axoneme. Rapid perfusion was used to minimize

---

**FIG. 9.** Shear angles (top) and sliding rates (calculated for doublets 7 and 8) of live bovine sperm. Shear angle measurements of live, actively beating sperm flagella were entered into a QuickBASIC program written to convert those values to the corresponding sliding rates (as described under Materials and Methods). Both shear angles (A) and sliding (B) are plotted at single scan intervals (1/60th sec) through the beat cycle of an intact (live) bull sperm.

**FIG. 10.** Shear angles (top) and sliding rates (calculated for doublets 7 and 8) of demembranated bull sperm reactivated with 1 mM ATP. Uninhibited, vigorously beating bovine sperm models were utilized to measure shear angles at 5-μm intervals along the flagellum. These values were subsequently converted to microtubule sliding rates with the assistance of the QuickBASIC computer program described under Materials and Methods. Both shear angles (A) and sliding (B) are plotted at single scan intervals (1/60th sec) through the beat cycle of a reactivated sperm model.
this effect; however, some underestimation of the sliding rate based on the inability to reach immediate, final ATP concentrations was unavoidable. Thus, the true equilibrium values would most likely be the highest individual rates. Fortunately, the two possible sources of error are not additive, but instead would cancel each other out.

The maximal sliding rate obtained at 1 mM ATP was approximately a factor of 2 higher than the 3.7 μm/sec reported by Si and Okuno (1993) for mouse sperm. In that study, the movement of the sheath toward the head was used to obtain a sliding rate. The axonemal elements remaining in the sheath were usually the 9, 1, 2 group, so head-ward movement of the sheath depended on the sliding of element 8 on 9, or 9 on 1. This movement would require considerable force development to slide the entire sheath (with any attached doublets) up along elements 9 and 1. It is also possible that species differences exist. Bull sperm microtubule sliding rates are lower than the 10- to 14-μm/sec velocities measured in simple sea urchin sperm flagella (Gibbons, 1975; Yano and Miki-Noumura, 1980; Oiwa and Takahashi, 1988), while mouse sperm, which are larger and more complex than bull sperm, present still slower sliding rates (3.7 μm/sec). Perhaps, as flagellar size increased, the accompanying dynein was modified. This could explain why larger sperm generally have a slower flagellar beat.

Several agents alter the motility of Triton X-100-extracted sperm models. Some (cAMP and theophylline) stimulate, some (KCN, Ni^{2+}, and VO_{3}^{−}) inhibit, and some (ADP) modify the flagellar beat. Direct observations were made of their effects on the motor mechanism.

The direct action of motility inhibitors (vanadate, and nickel) were assessed using the microtubule sliding assay. These agents had a surprisingly small impact on sliding rates. Vanadate is a potent inhibitor of sperm motility (Gibbons et al., 1978). Even at concentrations as low as 500 nM, VO_{3}^{−} blocks the flagellar beat of reactivated, demembranated bovine sperm. Dynein action is blocked when vanadate ion binds to the phosphate site that normally binds ATP (Sale and Gibbons, 1979). Vanadate does not compete with ATP for that site, and is thus a noncompetitive inhibitor. Vanadate allows the ATP-mediated relaxation of rigor waves, but will not relax rigor waves by itself (Mitchell and Warner, 1980). This suggests that, in the presence of ATP, vanadate causes the dynein to "pile up" in the relaxed (unbridged) state. Thus, vanadate could directly interrupt the cross-bridge cycle of the dynein motor and thereby interfere with the doublet-doublet sliding interaction. Sliding rates declined when relatively high (>5 μM) concentrations of vanadate were present. However, the most significant (>50%) reduction in sliding rate occurred only when the vanadate concentration exceeded that needed to block motility by a factor of 10 or more.

The divalent cation, Ni^{2+}, is a competitive inhibitor of motility. Larsen and Satir (1991) reported that both 22S and 14S dynein support translocation, yet 22S dynein translocates microtubules up to three times faster than 14S dynein. Adding Ni^{2+}, at concentrations that completely suppress the flagellar beat, stops all 14S dynein translocation, yet only partially inhibits translocation by the 22S dynein. Lindemann et al. (1995) reported that nickel selectively inhibits the dynein bridges on one side of the axoneme, causing a loss of motile force for one phase of the beat cycle, arresting motility with the flagellum in a curved configuration. However, nickel-inhibited flagella could resume spontaneous beating when mechanically bent in the direction opposite the resting curvature. This led to the deduction that Ni^{2+} discriminately inhibits the dynein arms on doublets 1, 2, 3, and 4 and thus the extrusion of the 9, 1, 2 bundle.

In other words, sliding rates observed in the presence of Ni^{2+} assess the relative impact of Ni^{2+} on only those bridges that remain active in the Ni^{2+}-inhibited flagellum. An earlier study showed that the sliding of elements 9, 1, and 2 out of the axoneme is strongly inhibited by 0.6 mM NiSO_{4} (Kanous et al., 1993). In the present study, adding 0.6 mM nickel decreased the rate of microtubule sliding induced by the remaining active bridges (between doublets 6 and 7, and between doublets 7 and 8) by only ~30% from controls cells.

Theophylline and KCN/2-DOG influence flagellar motility through the cAMP–kinase-A pathway. Reactivated sperm models, produced from theophylline-pretreated live sperm, are maximally activated. Reactivated sperm, produced from KCN/2-DOG-pretreated live sperm, are immotile or poorly motile. Methylxanthines cause cells to build up internal cAMP stores, while inhibition of oxidative respiration decreases cAMP production (Lindemann, 1978; Schoff and First, 1995). In spite of the striking difference in motility induced by these two treatments, the microtubular sliding rates only differed by approximately 40%. This supports Brokaw’s (1987) conjecture that “the primary effect of cAMP-dependent phosphorylation appears to be activation of a regulatory mechanism controlling flagellar oscillation rather than activation of the active sliding mechanism.” Stephens and Stommel (1989) contend that there is no clear evidence that phosphorylation directly influences dynein ATPase or microtubule sliding. Instead, they credit phosphorylation (in conjunction with calcium concentration) with regulat-
ing the switching mechanism that coordinates sliding. This leads to the proposition that cAMP (and calcium) sets or shifts the switch point for microtubule sliding, as opposed to controlling dynein activity. Thus, it is not surprising that theophylline and KCN\2-DOG had major effects on motility and less effect on microtubule sliding. Note that this study only evaluated sliding at the 7–8 (or 6–7) interdoublet bridges. It is conceivable that sliding between other doublet pairs may be selectively regulated by factors such as cAMP and Ca\textsuperscript{2+}. That possibility remains to be investigated in future studies.

ADP affects sperm flagellar beat frequency by its action on the mechanochemical cycle of dynein. Motility is altered when ADP competes for the phosphate site that normally binds ATP (Okuno and Brokaw, 1979) and when high concentrations of exogenous ADP hinder the release of ADP from the phosphate site following ATP hydrolysis (Omoto, 1989). The microtubule–dynein–ADP complex generates the force necessary for microtubule sliding. Thus, additional ADP prolongs the force generation step, leading to a greater beat amplitude and a dramatic change in the beat pattern of reactivated sperm flagella. While ADP did not inhibit motility, it had the greatest impact on sliding rates of any tested agent. This reinforces the idea that microtubule sliding rate and flagellar motility capability are not analogous. It also supports the contention that ADP acts by lengthening the duty cycle of the dynein (Omoto, 1989).

The interdoublet sliding rates, determined by the mitochondrial stripping method, were surprising. It appears possible that even a small number of working bridges can provide near maximal sliding rates. This has been suggested by in vitro studies of microtubule sliding on dynein-coated glass coverslips where the number of dyneins is small (Paschal et al., 1987). Supportive evidence was also derived from the results with high concentrations of VO\textsubscript{3}\textsuperscript{–} where the sliding rate decreased, the number of disintegrating cells was much more drastically reduced, and the number of subsequent splits from the initial “vanadate loops” dropped dramatically. The initial splitting of the axoneme, and the further splitting of the emerging bundle, both require the development of a critical level of force. The decrease in flagellar disintegration of VO\textsubscript{3}\textsuperscript{–}-treated cells may indicate that the total force was much more reduced by vanadate than the microtubule sliding rate would suggest.

In conclusion, interdoublet sliding rates measured in disintegrating flagella are adequate to account for the sliding that occurs in beating flagella during normal motility. It is also clear that greatly reducing the sliding rate (as seen with 4 mM ADP) does not interfere with the coordination of beating in intact flagella. Additionally, some potent inhibitors of motility (vanadate and Ni\textsuperscript{2+}) do not block or markedly impair the sliding mechanism (as measured by sliding rate only). Furthermore, strong stimuli to motility (such as cAMP) do not substantially increase microtubule sliding rates.

The authors thank Todd Miller for help with thin-sectioning and staining for the TEM figures. This report was supported by NSF Grant MCB 9220910.

REFERENCES


Sale, W. S., and Gibbons, I. R. (1979) Study of the mechanism of...


