ABSTRACT Flagellar beating of hyperactivated golden hamster spermatozoa was analyzed in detail using digital image analysis and was compared to that of nonhyperactivated (activated) spermatozoa in order to understand the change in flagellar beating during hyperactivation and the active microtubule sliding that brought about the change in flagellar beating. Hyperactivated flagellar beating, which was characterized by a sharp bend in the proximal midpiece and low beat frequency, was able to alter the waveform with little change in beat frequency (constant-frequency beating), whereas activated flagellar beating, which was characterized by a slight bend in the proximal midpiece and high beat frequency, was able to alter beat frequency with little change in the waveform (constant-curvature beating). These results demonstrate that flagellar beating of hyperactivated and activated spermatozoa were essentially different modes and that hyperactivation was the mode conversion from constant-curvature beating to constant-frequency beating. Detailed analysis of flagellar bends revealed that the increase in curvature in the proximal midpiece during hyperactivation was due to the increase in total length of microtubule sliding in a nearly straight region between bends, while the rate of microtubule sliding remained almost constant. Mol. Reprod. Dev. 73: 1412–1421, 2006. © 2006 Wiley-Liss, Inc.

Key Words: beat frequency; capacitation; flagellar bends; hamster spermatozoa; microtubule sliding

INTRODUCTION

Mammalian spermatozoa exhibit sequential changes in their motility during the capacitation in the female reproductive tract. Noncapacitated (activated) spermatozoa swim in relatively straight paths, whereas capacitated spermatozoa swim vigorously in circular paths or display nonprogressive figure-of-eight paths. This change in sperm motility during capacitation is termed hyperactivation (Yanagimachi, 1994). Hyperactivation seems to be beneficial for migration of spermatozoa in the female reproductive tract and for passage through the zona pellucida (Suarez and Dai, 1992; Stauss et al., 1995) and is, therefore, thought to be a crucial phenomenon for fertilization.

The change in sperm motility during hyperactivation is also important for understanding the mechanism regulating flagellar movement of spermatozoa because not only beat frequency, but also flagellar waveform remarkably change. Furthermore, motility parameters such as beat frequency, curvature, and waveform of flagellar beating are significantly correlated in the mammalian spermatozoa, implying that beat frequency and waveform of flagellar beating in mammalian spermatozoa appear to be regulated dependently (Ishijima et al., 2002, 2006). On the other hand, these parameters obtained from studies with echinoderm spermatozoa seem to be regulated independently (Gibbons, 1975), even though the echinoderm sperm flagella usually do not dramatically change their flagellar waveform. The difference in the structure between mammalian and echinoderm spermatozoa, for example, the presence of elastic outer dense fibers in the mammalian spermatozoa, may account for these differences in flagellar beating. To resolve the apparent discrepancy in movement characteristics between mammalian and echinoderm spermatozoa, we regarded it important to quantitatively analyze the oscillation characteristics of hyperactivated sperm flagella; for this purpose, the hyperactivated flagellar beating as well as the activated flagellar beating were examined using digital image analysis.

The driving force of the flagellar beating of spermatozoa is due to the active sliding between doublet...
microtubules in the axoneme (Summers and Gibbons, 1971; Gibbons, 1981). Therefore, the active microtubule sliding must behave in different ways to create different flagellar beating. To understand the change in flagellar beating based on the active microtubule sliding, various types of flagellar beatings of echinoderm spermatozoa have been analyzed by evaluating shear strain on a flagellum and were found to correspond to several patterns of active microtubule sliding (Gibbons, 1982; Ohmuro et al., 2004). This method has also been applied to the flagellar movement of mouse spermatozoa in order to examine the behavior of active microtubule sliding during bend growth and propagation (Woolley and Vernon, 2002).

In the present study, flagellar beatings of activated and hyperactivated golden hamster spermatozoa were analyzed quantitatively using digital image analysis in order to clarify the change in flagellar beating and the active microtubule sliding that causes these changes. Detailed analyses of flagellar beatings revealed that there were two modes: (1) a large change in beat frequency with little change in waveform, which was typically observed in activated flagellar beating, and (2) a large change in waveform with little change in beat frequency, which typically was observed in hyperactivated flagellar beating. The mode conversion during hyperactivation was caused not by the change in the sliding velocity of the microtubules but by the increase in the duration of the active microtubule sliding.

**MATERIALS AND METHODS**

**Sperm Preparations**

Spermatozoa obtained from sexually mature male Syrian golden hamsters (Mesocricetus auratus) were prepared as described by Ishijima et al. (2002). Briefly, male Syrian golden hamsters maintained in a light-controlled room (14L: 10D) were killed by CO2 inhalation, and their caudal epididymides were excised. In a Eppendorf tube, a mass of semen squeezed from the caudal epididymis was gently overlaid with 1-ml of warm (37°C) capacitation medium (100 mM NaCl, 5 mM KCl, 2.4 mM CaCl2, 0.49 mM MgCl2, 0.36 mM NaH2PO4, 24.9 mM NaHCO3, 0.006% penicillin, 1.2% BSA, 0.006% penicillin G, pH 7.5, 290–310 mOsm) completed by addition of hypotaurine (100 μM), epinephrine (1 μM), and penicillamine (20 μM). After incubation for 10 min at 37°C, the highly motile spermatozoa that appeared in the upper 0.5 ml of the sperm suspension were transferred into another Eppendorf tube, and the concentration of spermatozoa was adjusted to 2 × 10⁶ sperm/ml by addition of warm capacitation medium.

**Observation and Recording**

At various times after incubation, aliquots of 10 μl of sperm suspensions were placed on prewarmed slides and immediately covered by coverslips. The slides and coverslips had been coated with agarose to prevent sperm sticking. Movements of the sperm and their flagella were observed in the capacitation medium.

The movements of sperm and their flagella were observed using a Nikon Eclipse E600 microscope (Nikon Corp., Tokyo, Japan) with a phase-contrast condenser and a 20x BM objective, which produced bright images on a gray background. The temperature of the chamber was maintained at 37°C using a glass heating plate (ThermoPlate, Tokai Hit Co., Ltd., Shizuoka-ken, Japan) built in the microscope stage. Images obtained were captured directly to disk on a computer using a high-speed camera (HAS-200R, Ditect Co., Ltd., Tokyo, Japan), image software (Dipp-Motion 2D, Ditect Co., Ltd.), and a frame grabber (HAS-PCI, Ditect Co., Ltd.) at the rate of 200 images/sec. Alternatively, they were first recorded on Super-VHS 1/2-inch cassette videotape with a Dage-MTI 72 CCD video camera (Dage-MTI, Inc., Michigan City, IN) and a Panasonic AG 7350 videocassette recorder (Panasonic Broadcast and TV, Secaucus, NJ) linked to a video timer (Model VTG-33, For-A Corp., Cypress, CA), then captured to memory in the PC using the Dipp-Motion 2D and a frame grabber (SIM-PCI, Ditect Co., Ltd.) at the rate of 60 images/sec in order to reanalyze the images taken during our previous experiments (Ishijima et al., 2002).

**Analysis of Flagellar Beating**

For detailed field-by-field analysis, images of freely swimming spermatozoa were analyzed using image analysis software (Boohboh, BohbohSoft, Tokyo, Japan; Baba and Mogami, 1985). Linearity was defined as the ratio of the straight-line distance between the first and the last points of the center of the sperm head to the total length covered by the center of the head during a complete beat cycle. An individual flagellar image was tracked automatically using the Autotrace module of Bohboh software (Baba and Mogami, 1985; Ishijima et al., 2002; Ohmuro et al., 2004); the coordinate values of the flagellar shaft from the head-midpiece junction to the flagellar tip were obtained with reference to the direction of the axis of the sperm head (Fig. 1a). More than 26 successive images of a flagellum (more than one beat cycle) were analyzed to obtain accurate values of the motility parameters. The angle of the tangent to the flagellar shaft with reference to the sperm head axis (this angle will be referred to as the shear angle) and the curvature of the shaft were calculated from the coordinate values obtained as functions of the distance along the flagellum from the head-midpiece junction (Fig. 1b) and time (Fig. 1c). We will refer to the flagellar bend having the same sense as the curvature of the sperm head known as the “pro-hook bend” and the opposite bend known as the “anti-hook bend” (Ishijima et al., 2002). The sign of the shear angle and curvature was set so that the curvature of the pro-hook bend might be positive.

Beat frequency (f) was calculated from the period required for one complete beat cycle, which was obtained from the time at which the course of curvature and mean curvature intersected. The propagation velocity of...
the flagellar bend was determined from the distance along the flagellum and the time of a peak of the pro-hook bend in the proximal midpiece that was the basal half-wavelength region from the head-midpiece junction, because in this region, flagellar beating was remarkably altered during hyperactivation (Ishijima et al., 2002). The wavelength was calculated from the ratio of propagation velocity to beat frequency. The mean curvature of the proximal midpiece \( (\kappa_{pm}) \) was obtained by averaging all means of absolute values of curvature of the pro-hook and the anti-hook bends at points in the proximal midpiece over this region. The maximum shear angle \( (\theta_{\text{max}}) \) was the maximum amplitude of shear angle curves in the region between 0.50 and 0.75 wavelength from the head-midpiece junction (Fig. 1b). The sliding velocity of the microtubules, which is proportional to the rate of the shear angle, was determined by \( 4\theta_{\text{max}} \) (Gibbons, 1982).

To clarify the change in curvature and shear angle of flagellar bends along the flagellum as \( \kappa_{pm} \) changed, pseudo-color plots of the curvature and the shear angle of flagellar bends were created; first, the relationships between the amplitude of curvature curves and shear angle curves at each point on the flagellum and \( \kappa_{pm} \) were obtained after fitting a quartic function to the data. The color corresponding to the value of the amplitude of curvature curves and shear angle curves was then arranged at each point on a coordinate system comprised of two axes of distance from the head-midpiece junction and of \( \kappa_{pm} \). Finally, this procedure was carried out from the head-midpiece junction to the flagellar tip, and contour lines were drawn on the pseudo-color map.

Data were analyzed with one-way ANOVA using SPSS 11.0J (SPSS Japan Inc., Tokyo, Japan). The significant level was considered to be \( P < 0.05 \). The correlation coefficient \( (r) \) was used to examine the relationships among motility parameters.

**RESULTS**

**Oscillation Characteristics of Flagellar Beatings**

Hamster spermatozoa immediately after dilution in the capacitation medium swam in relatively straight paths with flagellar beating consisting of small bends in the proximal midpiece with a high beat frequency. With the extended incubation times, spermatozoa began to exhibit nonprogressive, whiplash movements with flagellar beating of sharp bends in the proximal midpiece and low beat frequency (Fig. 2). Thus, the most essential change in waveform during hyperactivation was the increase in curvature of the flagellar bends in the proximal midpiece, especially in the basal half-wavelength region from the head-midpiece junction (Fig. 3; Ishijima et al., 2002). Therefore, the mean curvature of the proximal midpiece \( (\kappa_{pm}) \) was used as an index of waveform of the hyperactivated flagellar beating. To clarify the change in flagellar bends along the flagellum during hyperactivation, the amplitude of curvature curves was plotted against \( \kappa_{pm} \) and the distance from the head-midpiece junction (Fig. 4a). Because the contour lines in the proximal midpiece ran relatively horizontally and the color changed from cold to warm with increasing \( \kappa_{pm} \) (Fig. 4a), the curvature of flagellar bends in this region increased with \( \kappa_{pm} \). On the other hand, the contour lines in the distal region of a flagellum ran perpendicularly, and the color showed little change with increasing \( \kappa_{pm} \), indicating that the curvature in the distal regions remained almost constant. It was primarily in the proximal midpiece that the
Curvature of the flagellar bends increased, accompanied by a change in sperm motility during hyperactivation. Based on incubation time in the capacitation medium and $k_{pm}$, which was sufficient to define the change in flagellar bends during hyperactivation as mentioned above, spermatozoa were classified into activated, transient, and hyperactivated spermatozoa: the cells that had been incubated less than 15 min and had a $k_{pm}$ of not more than 0.018 $\mu$m were regarded as activated spermatozoa (a, b), spermatozoa incubated more than 2 hr and having a $k_{pm}$ not less than 0.030 $\mu$m were regarded as hyperactivated spermatozoa (d, e), and the remainder was regarded as transient spermatozoa (c). Bar = 100 $\mu$m.

A plot of beat frequency against $k_{pm}$ demonstrated that the beat frequency of flagellar beating changed in a biphasic manner: one phase of sharp reduction up to about 8 Hz, followed by a phase of gradual decrease. The first phase mainly consisted of the activated flagellar beating, and the second phase contained the transient and hyperactivated flagellar beatings. These results, therefore, revealed that hyperactivation was a mode of conversion from the beating mode of relatively constant-frequency to the beating mode of relatively constant-curvature that was mainly exhibited in the activated flagellar beating to the beating mode of relatively constant-curvature that was observed in the transient and hyperactivated flagellar beatings. The essentially similar biphasic characteristic was also found in the relationship between propagation velocity and $k_{pm}$.
of the flagellar bend and $\kappa_{\text{pm}}$ (Fig. 6b); namely, the propagation velocity of flagellar bends in the activated flagellar beating declined rapidly up to about 0.8 mm/sec, whereas the propagation velocity in the transient and the hyperactivated flagellar beatings showed little change with $\kappa_{\text{pm}}$. The biphasic nature of the flagellar beatings was further verified by the relationships between other motility parameters (Fig. 7). Data for beat frequency and wavelength were distributed along different lines (Fig. 7a), demonstrating that there were two relationships between beat frequency and wavelength. In addition, the relationship between propagation velocity and beat frequency was not unique (Fig. 7b).

**Microtubule Sliding of Flagellar Beatings**

Active sliding between outer doublet microtubules exerts the driving force of flagellar beating. The microtubule sliding, therefore, must be changed during hyperactivation. To examine the microtubule sliding during hyperactivation, shear angle curves were determined since the magnitude and rate of change of the shear angle are proportional to the extent of microtubule sliding and the sliding velocity, respectively.

The shear angle of the activated and hyperactivated flagellar bends are compared in Figure 8. The amplitude of the shear angle curves of hyperactivated flagellar bends was, on the whole, larger than that of the activated ones, but its increase was remarkable at the region between 0.50 and 0.75 wavelength from the head-midpiece junction. To clarify the change in microtubule sliding along the flagellum during hyperactivation, the amplitude of the shear angle curves was plotted against $\kappa_{\text{pm}}$ and the distance from the head-midpiece junction (Fig. 4b). The contour lines ran relatively horizontally, except for the proximal and distal regions of a flagellum, and the color changed from cold to warm with increasing $\kappa_{\text{pm}}$, the shear angle increased with $\kappa_{\text{pm}}$, especially at the proximal and distal regions.
region between 0.50 and 0.75 wavelength from the head-midpiece junction. Thus, the amount of microtubule sliding at this region increased remarkably with the increase in $k_{pm}$. This relationship was clearly indicated by the high correlation coefficient between the maximum shear angle, which is proportional to the amount of microtubule sliding, and $k_{pm}$ ($r = 0.96$) (Fig. 9a). These analyses demonstrated that the increase in the amount of microtubule sliding at a nearly straight region between bends was responsible for the increase in curvature of growing bends at the midpiece during hyperactivation. On the other hand, the amplitude of the shear angle curves at the proximal and distal regions of a flagellum changed little during hyperactivation, suggesting that the amount of microtubule sliding also changed little at both regions. The rate of microtubule sliding at the region between 0.50 and 0.75 wavelength from the head-midpiece junction showed no correlation with $k_{pm}$ ($r = 0.17$) (Fig. 9b), and further, there was no significant difference in the sliding velocity between activated and hyperactivated flagellar beatings ($r = 0.30$). The constant rate of microtubule sliding in the activated and hyperactivated flagellar beatings was also clearly shown by the linear relationship between the maximum shear angle and the beat period of flagellar beating (Fig. 10).

These analyses revealed that the change in flagellar beating during hyperactivation was caused not by the change in sliding velocity but by increases in the amount of microtubule sliding at a nearly straight region between the bends of a flagellum or by an increase in the period of flagellar beating.

Fig. 6. Relationships of beat frequency and propagation velocity of flagellar bends to $k_{pm}$. (●) The activated, (□) transient, and (■) hyperactivated flagellar beatings.

Fig. 7. Relationships of wavelength and propagation velocity of flagellar bends to beat frequency. (●) The activated, (□) transient, and (■) hyperactivated flagellar beatings. Wavelength and beat frequency are plotted on a double logarithmic scale (a). Lines were fitted by least-squares methods for each separated group. a: Solid line for the activated flagellar beating was given by $\lambda = 490 v (f^{-0.58})$, and the dotted line for the transient and hyperactivated flagellar beatings was given by $\lambda = 280 v (f^{-0.50})$, where $\lambda$ is wavelength and $f$ is beat frequency of the flagellar beatings. b: Solid line for the activated flagellar beating was given by $V = \lambda f = 490 f^{0.42}$, and dotted line for the transient and hyperactivated flagellar beatings was given by $V = 280 f^{0.50}$, where $V$ is the propagation velocity of flagellar bends.
DISCUSSION

There were three important findings in the present study: (1) flagellar beatings of hamster spermatozoa consisted of two beating modes; a nearly constant-curvature mode observed mainly in activated spermatozoa and a nearly constant-frequency mode in hyperactivated spermatozoa; (2) these beating modes were two different aspects of the constant-rate of microtubule sliding in the flagellar beating; (3) hyperactivation, a mode conversion of nearly constant-curvature beating to nearly constant-frequency beating, was caused by an increase in the amount of microtubule sliding at a nearly straight region between bends.

Two Beating Modes in Hamster Sperm Flagella

It is well known that activated spermatozoa beat with relatively constant waveform while their beat frequency widely changes (Ishijima and Mohri, 1990). Temperature easily changed the beat frequency of flagellar beating with little change in the waveform (Holwill, 1966; Katz and Overstreet, 1979; Rikmenspoel, 1984). This feature in activated flagellar beating was further confirmed by permeabilized spermatozoa; namely, the beat frequency of demembranated, reactivated spermatozoa widely varied with the concentration of MgATP$^{2-}$, while the flagellar waveform was maintained relatively constant (Ishijima and Witman, 1987). These features of flagellar beatings were extensively studied in echinoderm spermatozoa (Gibbons, 1982; Brokaw, 1989), and thereby, it was presumed that the beat frequency and waveform of flagellar beating were independently regulated (Gibbons, 1975).

On the other hand, flagellar beating of hyperactivated spermatozoa has been predicted to include the existence of an entirely different mode of flagellar beating (Ishijima and Mohri, 1990) because the flagellar waveform of hyperactivated spermatozoa changes widely without a change in beat frequency, which is the so-called isochronism of oscillations (the period of oscillation is independent of amplitude). Detailed analysis of hyperactivated flagellar beating revealed that the fundamental change in flagellar waveform was due to an increase in curvature at the proximal midpiece while the beat frequency of flagellar beating remained almost constant.

Fig. 8. Difference in shear angle of flagellar bends between activated and hyperactivated flagellar beatings. The activated (a) and hyperactivated (b) flagellar beatings. Shear angle was determined using Bohboh software as a function of distance from the head-midpiece junction along a flagellum in the same spermatozoa shown in Figure 2a and d. Bars represent the regions between 0.5 and 0.75 wavelength from head-midpiece junction.

Fig. 9. Microtubule sliding as the mean curvature of the proximal midpiece ($\kappa_{pm}$) changed. (●) The activated, (□) transient, and (■) hyperactivated flagellar beatings. a: Maximum shear angle that is proportional to the amount of microtubule sliding increased with $\kappa_{pm}$. b: Sliding velocity changed little as $\kappa_{pm}$ increased; sliding velocity was $67.4 \pm 9.9$ µm/sec (mean ± SD; average of 11 sperm) for activated flagellar beating and $64.1 \pm 9.6$ µm/sec (average of 21 sperm) for hyperactivated flagellar beating.
activated spermatozoa, which is a critical parameter in In fact, the propagation velocity of flagellar bends of of producing high-speed swimming of the spermatozoa. waveform in activated spermatozoa has the advantage frequency of flagellar beatings with nearly constant logical significance of these beating modes. High beat found in the present study may determine the physio-

Independent regulation of beat frequency and waveform almost independently varied wavelength from the head-midpiece junction and was primarily increased in the region between 0.50 and 0.75 activation, the amplitude of the shear angle curves increased over the whole length of the flagellum, but whereas that of the hyperactivated flagellar beating was about 7 Hz (Ishijima et al., 2002). Propagation velocity varied by, at most, 30% at a sampling rate of 60 Hz. However, these errors essentially did not influence the results; namely, the data for the activated flagellar beating lay on a regression line that differed from that of hyper-

Molecular Reproduction and Development. DOI 10.1002/mrd

CONSTANT SLIDING VELOCITY IN FLAGELLAR BEATING 1419

constant (Ishijima et al., 2002, 2006). These results demonstrate that flagellar beating in hyperacti-

spermatozoa is different from that in activated spermatozoa. In our previous studies (Ishijima et al., 2002, 2006), one of the authors has reported that beat frequency and wavelength of flagellar beating in activated and hyper-

activated golden hamster and monkey spermatozoa were significantly correlated, and therefore, the regula-

tions of beat frequency and waveform of flagellar beating appeared to be dependent. As mentioned above, the present detailed analyses of motility parameters also revealed that there were two different relationships between beat frequency and waveform: nearly constant-waveform beating in activated spermatozoa and nearly constant-frequency beating in hyperactivated spermatozoa. Thus, the regulation of beat frequency and waveform is fundamentally dependent, although beat frequency and waveform almost independently varied because the spermatozoa beat in either of the two modes. Independent regulation of beat frequency and waveform predicted from flagellar beatings in echinoderm spermatozoa presumably corresponds to the constant-waveform beating in the activated spermatozoa in present study. On the other hand, constant-frequency beating mode observed in hyperactivated spermatozoa has not been reported in echinoderm spermatozoa to date (Brokaw, 1989). Oscillation characteristics of the two beating modes found in the present study may determine the physio-

logical significance of these beating modes. High beat frequency of flagellar beatings with nearly constant waveform in activated spermatozoa has the advantage of producing high-speed swimming of the spermatozoa. In fact, the propagation velocity of flagellar bends of activated spermatozoa, which is a critical parameter in evaluating the limit of swimming speed of the sperma-

tozoa (Lighthill, 1976), was more than twice that of flagellar bends of the hyperactivated spermatozoa (Fig. 6b). On the other hand, the low beat frequency of flagellar beatings with sharp bends in the hyperacti-

vated spermatozoa is useful for generating the large thrust required for passage through the zona pellucida (Drobnis et al., 1988). Therefore, the conversion of the nearly constant-curvature beating mode to the nearly constant-frequency beating mode fulfills these two requirements for accomplishing fertilization: the high-speed swimming in the activated spermatozoa and the large thrust in the hyperactivated spermatozoa. Access-

sory fibers such as outer dense fibers in the mammalian sperm flagella may be indispensable for generating the nearly constant-frequency mode in hyperactivated flagellar beatings because the high power strokes of the hyperactivated spermatozoa require a hard flagellum to beat continuously.

It is well known that the image-sampling rate affects several motility parameters (Owen and Katz, 1993; Mortimer and Swan, 1999). To estimate the relative effect of image sampling rate on motility parameters, the flagellar beating of activated and hyperactivated spermatozoa was recorded using a high-speed video system at the rate of 200 images/sec. We compared motility parameters obtained from analysis performed on every image with those from analysis of only every third image. There was no significant difference in both beat frequency and sliding velocity between the two sampling rates. Linearity was influenced by the sam-

pling rate; namely, the error was less than 20% if more than four images per beat cycle were recorded, which corresponded to beat frequencies of less than 15 Hz at a sampling rate of 60 Hz (mean beat frequency of the activated flagellar beating was about 15 Hz, whereas that of the hyperactivated flagellar beating was about 7 Hz; Ishijima et al., 2002). Propagation velocity varied by, at most, 30% at a sampling rate of 60 Hz. However, these errors essentially did not influence the results; namely, the data for the activated flagellar beating lay on a regression line that differed from that of hyper-

activated flagellar beating (data not shown).

Microtubule Sliding in Hamster Sperm Flagella

To understand the active microtubule sliding that induces the hyperactivated flagellar beating, the shear angle that is proportional to the amount of microtubule sliding was measured in the flagellar bends of activated and hyperactivated spermatozoa. Following hyperacti-

vation, the amplitude of the shear angle curves increased over the whole length of the flagellum, but primarily increased in the region between 0.50 and 0.75 wavelength from the head-midpiece junction and was highly noticeable (Fig. 8). On the other hand, the amplitude of the shear angle curves at the proximal and the distal flagellum changed little during hyper-

activation (Fig. 4b), suggesting that the increases in the degree of microtubule sliding at a nearly straight region
between bends, induces the changes in flagellar beating of hyperactivated spermatozoa. Microtubule sliding in other regions of the flagellum appears to have a limited involvement in hyperactivation. At distal parts of the hyperactivated sperm flagellum, small waves were often superimposed on primary bends (data not shown), which are similar to the additional (secondary) waves analyzed by Woolley and Vernon (2002). These results suggest that active sliding between the doublet microtubules changes dynamically along a flagellum. The degree of microtubule sliding is the product of sliding velocity and duration; thus, duration of microtubule sliding lengthens during hyperactivation, which presumably causes the decrease in beat frequency. The amplitude of the shear angle curves increased sharply at a nearly straight region between bends, whereas the curvature dramatically increased at the basal half-wavelength region from the head-midpiece junction of hyperactivated sperm flagella. The difference in the location of these changes is possibly explained by the mechanism of bend formation by microtubule sliding (Satir, 1968; Shingyoji et al., 1977).

Hyperactivation has not been defined well. A spermatozoon exhibiting very vigorous, whiplash-like flagellar beatings are usually considered to be hyperactivated, however, these beating patterns may not be observed in spermatozoa in all species (Yanagimachi, 1994), since mechanical properties of the sperm flagellum differ in spermatozoa of different species. We found in the present study that the sliding velocity remained almost constant in flagellar beating of both activated and hyperactivated spermatozoa. This feature of the active microtubule sliding should be incorporated into the criteria used to describe hyperactivation since the constant rate of microtubule sliding seems to be a fundamental property in flagellar beating of the spermatozoa.

Mechanism Inducing Hyperactivated Flagellar Beating

Hyperactivation is a mode conversion of nearly constant-curvature beating to nearly constant-frequency beating, which results from the nearly constant-rate of microtubule sliding. Therefore, decreases in beat frequency and increases in shear angle of the activated flagellar beating are necessary for successful hyperactivation. Much evidence indicates that elevated intracellular Ca$$^{2+}$$ increases flagellar bending (Lindemann et al., 1987; Lindemann and Goltz, 1988; Ho et al., 2002; Ishijima et al., 2006). Thus, large bends of hyperactivated flagellar beating in the proximal midpiece is most likely caused by increases in intracellular Ca$$^{2+}$$ concentrations. However, decreases in beat frequency during hyperactivation cannot be explained by increases in Ca$$^{2+}$$ concentration (Lindemann et al., 1987; Ishijima et al., 2006), since increases in shear angle may result in decreases in beat frequency if the sliding velocity of the microtubule is constant. In this regard, movement characteristics of transient flagellar beating may provide an important clue; sliding velocity of the transient flagellar beating was somewhat low compared to that of both activated and hyperactivated flagellar beatings (Fig. 9); that is, beat period and maximum shear angle of the transient flagellar beating were significantly small compared to those of hyperactivated flagellar beating (Fig. 10). Perhaps, cAMP increases the maximum shear angle and beat period since cAMP is known to increase the curvature of flagellar bends in the proximal midpiece, and decreases beat frequency when spermatozoa are demembranated and reactivated in a moderate solution containing high levels of cAMP (Ishijima et al., 2006).

Changes in stiffness of the accessory fibers in mammalian spermatozoa may also be involved in inducing hyperactivation. However, various types of experiments have suggested that the accessory fibers in the mammalian spermatozoa do not play an active role in flagellar beating (Baccetti et al., 1973; Hinsch et al., 2004) and do not change its mechanical properties. Recent experiments using permeabilized rat spermatozoa (Schmitz-Lesich and Lindemann, 2004) strongly support this idea; that is, a strong bend in the proximal midpiece, whose curvature was similar to that of flagellar bends in hyperactivated spermatozoa, is a result of only the action of the dyneins.

The finding in the present study that hyperactivated flagellar beating is a different mode from activated flagellar beating, is very important for clinical aspects since the hyperactivated spermatozoa may be accurately separated from activated spermatozoa using motility parameters, for example, swimming speed, linearity, and beat frequency. Accurate detection of hyperactivated spermatozoa is essential for understanding of the basic mechanisms of fertilization and successful treatment of infertility (Burkman, 1990). Once the critical motility parameters, which accurately select hyperactivated spermatozoa, are established in a computer-aided sperm analysis system, accurate determination of the numbers of hyperactivated spermatozoa is possible in real time, resulting in the determination of the specific role(s) of hyperactivation in fertilization.

ACKNOWLEDGMENTS

We are grateful to Gary Cherr for critically reading the manuscript.

REFERENCES


Ho HC, Granish KA, Suarez SS. 2002. Hyperactivated motility of bull sperm is triggered at the axoneme by Ca$^{2+}$ and not cAMP. Dev Biol 250:209–217.


