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EFFECT OF BEAT FREQUENCY ON THE VELOCITY OF MICROTUBULE SLIDING IN REACTIVATED SEA URCHIN SPERM FLAGELLA UNDER IMPOSED HEAD VIBRATION

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Summary

The heads of demembranated spermatozoa of the sea urchin *Tripneustes gratilla*, reactivated at different concentrations of ATP, were held by suction in the tip of a micropipette and vibrated laterally with respect to the head axis. This imposed vibration resulted in a stable rhythmic beating of the reactivated flagella that was synchronized to the frequency of the micropipette. The reactivated flagella, which in the absence of imposed vibration had an average beat frequency of 39 Hz at 2 mmol l^{-1} ATP, showed stable beating synchronized to the pipette vibration over a range of 20–70 Hz. Vibration frequencies above 70 Hz caused irregular, asymmetrical beating, while those below 20 Hz induced instability of the beat plane. At ATP concentrations of 10–100 μmol l^{-1}, the range of vibration frequency capable of maintaining stable beating was diminished; an increase in ATP concentration above 2 mmol l^{-1} had no effect on the range of stable beating. In flagella reactivated at ATP concentrations above 100 μmol l^{-1}, the apparent time-averaged sliding velocity of axonemal microtubules decreased when the imposed frequency was below the undriven flagellar beat frequency, but at higher imposed frequencies it remained constant, with the higher frequency being accompanied by a decrease in bend angle. This maximal sliding velocity at 2 mmol l^{-1} ATP was close to the sliding velocity in the distal region of live spermatozoa, possibly indicating that it represents an inherent limit in the velocity of active sliding. The results are consistent with the view that the sliding velocity of axonemal microtubules does not depend solely upon the local concentration of ATP, but is also dependent upon the oscillatory mechanism associated with initiation of new flagellar bends.

Key words: flagella, beat frequency, sliding velocity, sea urchin, *Tripneustes gratilla*.

Introduction

The movement of eukaryotic cilia and flagella is characterized by rhythmic generation of bending waves. It has been established that the motive force for the bending movement is derived from active sliding between the nine outer doublet microtubules of the axoneme (Satir, 1968; Summers and Gibbons, 1971; Shingyoji *et al.* 1977; Brokaw, 1989*a*). This active sliding is caused by ATP-driven cycling of the dynein arm crossbridges that extend from the A-tubule of each doublet towards the B-tubule of the adjoining doublet. In demembranated, trypsin-treated axonemes, the ATP-induced cycling of the dynein arm crossbridges causes longitudinal sliding between the outer doublet microtubules, resulting in disintegration of the axoneme into individual doublets – a process that can be directly observed under a dark-field microscope (Summers and Gibbons, 1971; Kamimura and Takahashi, 1981). In normally beating cilia and flagella, the sliding movement is regulated so that bending waves are continuously generated and propagated along the axoneme at regular intervals.

The mechanisms that regulate active sliding to generate normal flagellar and ciliary beating are poorly understood, but probably involve at least four levels of regulation (Gibbons, 1989; Brokaw, 1989*b*). The first involves the intrinsic ATP-driven oscillation of the dynein arms (Kamimura and Kamiya, 1992). A second system is presumably required to coordinate the activity of the dynein arms arranged along each doublet microtubule with the initiation and propagation of successive flagellar bends (Brokaw and Gibbons, 1973; Okuno and

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Hiramoto, 1976; Shingyoji *et al.* 1977; Takahashi *et al.* 1982). A third level, associated with the regulation of the pattern of active sliding around the axonemal axis, may constitute a switching mechanism that alternates activity between dynein arms located on opposite sides of the axonemes, so that the flagellum will form planar bends (Wais-Steider and Satir, 1979; Satir, 1985; Sale, 1986). The fourth level of regulation is responsible for the overall initiation of flagellar beating and, in some cases, involves phosphorylation of axonemal polypeptides (Brokaw, 1987).

Since the function of the dynein arms in the beating flagella appears to be both heterogeneous and complex, understanding the nature of the oscillation-generating system will require biophysical/biomechanical investigation at the organellar as well as the molecular level. There is ample evidence that mechanical and chemical conditions influence the frequency of the flagellar oscillation (Brokaw, 1977, 1980; Brokaw and Josslin, 1973; Brokaw and Simonick, 1977; Asai and Brokaw, 1980); for example, an increase in the viscosity of the medium causes a decrease in the beat frequency of both live and reactivated sperm flagella (Brokaw, 1966, 1975). However, no systematic investigation has been made so far on the effect of changing the beat frequency on other parameters of flagellar movement, because there has been no reliable method for reversibly increasing or decreasing the beat frequency.

We found that the flagellar beat frequency of live or reactivated sea urchin spermatozoa could be controlled reversibly by imposing vibration on their heads using a sinusoidally vibrating micropipette that holds the sperm head by gentle suction (Gibbons *et al.* 1987). This new technique also allowed us to manipulate the flagellar beat plane experimentally, as the beat plane was found always to coincide with the plane of vibration of the micropipette.

In previous studies (Gibbons *et al.* 1987; Shingyoji *et al.* 1991*a*), we showed that live sea urchin spermatozoa held by their heads in the tip of a vibrating micropipette can be forced to synchronize their flagellar beat with the imposed vibration. In this way, both the flagellar beat frequency and the sliding velocity of the microtubules could be modulated over a wide range without loss of either stability or regularity of the waveform. It was therefore suggested that the sliding velocity in normally beating flagella depends not only on 'local' variables, such as the ATP concentration, but also on other control mechanisms that are closely associated with the initiation of bending waves. The nature of such control mechanisms is still unknown. Therefore, it is important to study the effect of imposed vibration on the beating of demembranated flagella reactivated at various concentrations of ATP.

Reports on the relationship between the beat frequency of reactivated flagella and the concentration of ATP (Brokaw, 1967, 1975; Gibbons and Gibbons, 1972) have shown that it can be described by simple saturation kinetics (Michaelis–Menten kinetics). Similar kinetics have been obtained for the effect of ATP concentration on the velocity of sliding disintegration of protease-digested flagellar axonemes (Takahashi *et al.* 1982). Since a change in the concentration of ATP in the reactivating solution affects both the beat frequency and the sliding velocity, it is difficult to distinguish experimentally the mechanism regulating the beat frequency from that regulating sliding velocity (Brokaw, 1989*b*). Our procedure for changing the beat frequency by imposed vibration has an advantage in this respect. If the beat frequency were to be directly determined by the sliding velocity, which in turn was governed by the ATP concentration, one might expect the sliding velocity to remain unaffected by various imposed vibration frequencies, at a given concentration of ATP. We report here that this is generally not the case and that, at low ATP concentrations, the sliding velocity increased with the beat frequency whereas, at high ATP concentrations, the sliding velocity decreased with imposed beat frequency below the undriven value. Possible mechanisms by which the ATP concentration might determine the frequency of flagellar oscillation are discussed.

Materials and methods

Spermatozoa were obtained from the sea urchin *Tripneustes gratilla* by injecting 0.5 mol l^{-1} KCl into the body cavity. They were then diluted into Ca^{2+}-free artificial sea water containing 465 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 25 mmol l^{-1} $MgCl_2$, 28 mmol l^{-1} $MgSO_4$, 0.2 mmol l^{-1} EDTA and 2 mmol l^{-1} Tris–HCl (pH 8.2).

Reactivated spermatozoa were prepared under 'potentially symmetric' conditions (Gibbons and Gibbons, 1980) by adding two drops of the suspension of spermatozoa to 3 ml of demembranating solution containing 0.04 % (w/v) Chaps {3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate}, 0.1 % Nonidet P-40, 3 mmol l^{-1} $CaCl_2$, 0.1 mmol l^{-1} EGTA, 1 mmol l^{-1} dithiothreitol and 10 mmol l^{-1} Tris acetate (pH 8.2) for 45–60 s (Katada *et al.* 1989) at room temperature (24–26 °C). Extraction was stopped by dilution of five drops of the resultant suspension into 7 ml of a reactivating solution without ATP (0.2 mol l^{-1} potassium acetate, 2 mmol l^{-1} magnesium acetate, 0.1 mmol l^{-1} EGTA, 10 mmol l^{-1} Tris acetate, pH 8.2, and 1 mmol l^{-1} dithiothreitol). Just before each experiment, the demembranated spermatozoa were reactivated with a reactivating solution containing ATP at final concentrations between 10 μmol l^{-1} and 4 mmol l^{-1} (in the case of 4 mmol l^{-1} ATP, the concentration of magnesium acetate was increased to 4 mmol l^{-1}). All experiments were carried out at room temperature.

The experimental apparatus, including the optical and video systems, sperm micromanipulation and computer support, were the same as described previously (Eshel and Gibbons, 1989; Shingyoji *et al.* 1991*a*,*b*). The micropipette was vibrated laterally with a sinusoidal waveform of 16–26 μm peak-to-peak amplitude in a plane parallel to the glass slide and approximately 50 μm above it.

Flagellar images were traced from the monitor screen,

digitized and stored as values of angular orientation relative to the axis of the sperm head at 0.4 μm intervals along the length of the flagellum (Eshel and Gibbons, 1989). Bend angles were obtained by differentiating shear angle curves (curves of angles relative to the axis of small segments along the flagellum as a function of position along the flagellum) to obtain plots of flagellar curvature and then integrating positive or negative portions of the plots of curvature to obtain the area located between those portions and the axis of zero curvature (Eshel and Brokaw, 1987). We chose to represent the tubule sliding velocity at a given point on the flagellum by the product 2 $\times$ (beat frequency) $\times$ (averaged angle of principal and reverse bends centred at the point in question) (Brokaw, 1971; Gibbons, 1982; Takahashi *et al.* 1982; Shingyoji *et al.* 1991*a*; Brokaw, 1991). This product is proportional to the time-averaged speed of sliding between each pair of doublet microtubules around the axoneme, with the constant of proportionality differing for the various microtubule pairs. The measurements of shear angles in sea urchin sperm flagella can be inaccurate because of flexure at the head/tail junction (Brokaw, 1991). Such flexure, however, will not alter any of the conclusions reached about the average sliding velocities in the present work. The sliding velocity was calculated separately for the proximal and the mid-distal regions of the flagellum, with bend angles taken at 5–17 μm for the proximal and 25–30 μm for the mid-distal regions along the flagellum. The experimental errors introduced during the various stages of the analysis were estimated previously (Eshel and Gibbons, 1989).

The vibration frequencies were scanned up and down from the undriven beat frequency, i.e. the frequency of beat in the absence of imposed vibration at a given concentration of ATP, and data at frequencies that obtained stable beating were further analyzed. Four beat cycles were analyzed for each vibration frequency at each ATP concentration, with approximately 10 flagellar images for each beat cycle. Experiments were performed on 6–9 spermatozoa at each concentration of ATP, with three spermatozoa (that is, about 120 flagellar images) analyzed for each value of vibration frequency in live and reactivated spermatozoa, except in 10 μmol l^{-1} ATP, where two spermatozoa (80 flagellar images) were analyzed.

Results

Effect of ATP concentration on the stability of beating in reactivated sperm flagella

We have previously described the effect of lateral vibration on the frequency and stability of beating in live spermatozoa of the sea urchin *Hemicentrotus pulcherrimus* (Shingyoji *et al.* 1991*a*). As a different species of sea urchin was used in the reactivation experiments reported here, we first examined the responses of live spermatozoa of *Tripneustes gratilla* to imposed head vibration. We found that the spermatozoa, whose natural beat frequency was about 46 Hz, showed similar responses to lateral vibration, although the range of frequency capable of maintaining stable beating (30–75 Hz) was somewhat narrower than in the *H. pulcherrimus* spermatozoa (35–90 Hz).

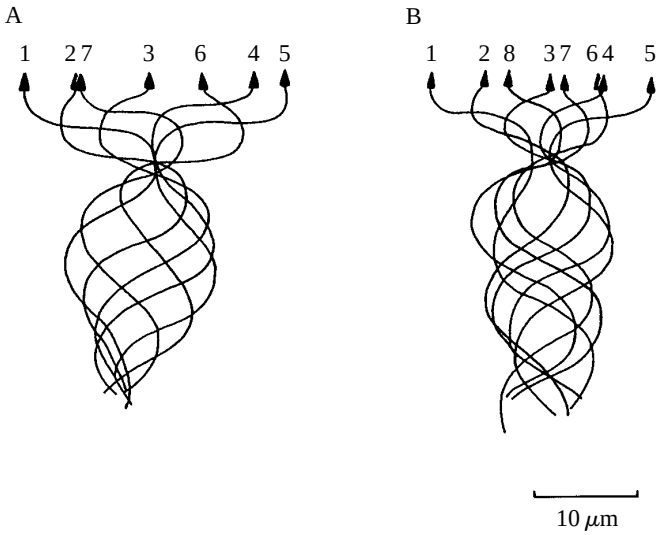

Fig. 1. Typical tracings of a live (A) and a reactivated spermatozoon (B) during lateral vibration. (A) Vibration at 50 Hz. The natural beat frequency was 48 Hz. (B) 2 mmol l^{-1} ATP. Vibration at 45 Hz. The undriven beat frequency was 37 Hz.

In reactivated spermatozoa, lateral vibration of the pipette with frequencies different from the undriven frequency caused the flagella to beat synchronously with the movement of the pipette, within a certain range of frequencies. The pipette vibration also caused the plane of flagellar beating to coincide with that of pipette vibration (Gibbons *et al.* 1987; Shingyoji *et al.* 1991*b*). Fig. 1A shows typical tracings of a live sperm flagellum and Fig. 1B shows a reactivated flagellum at 2 mmol l^{-1} ATP beating stably at vibration frequencies (50 Hz for live spermatozoa and 45 Hz for reactivated spermatozoa) above their natural or undriven beat frequency (48 Hz for live spermatozoa and 37 Hz for reactivated spermatozoa).

The effect of lateral vibration on reactivated flagella was studied at ATP concentrations over a range of 10 μmol l^{-1} to 4 mmol l^{-1} with vibration frequencies of 5–80 Hz and vibration amplitudes of 16–26 μm. Fig. 2 summarizes the effect of vibration frequency and ATP concentration on the stability of beating. The range of vibration frequency capable of maintaining stable beating increased gradually with ATP concentration up to 100 μmol l^{-1}. At higher ATP concentrations, stable beating was obtained at frequencies between about 20 and 70 Hz. Vibration frequencies below 20 Hz caused unstable beating with instability of the beat plane, and at frequencies above 70 Hz the flagella beat with irregular asymmetric waveforms. Similar unstable beating patterns were also observed at lower ATP concentrations. Unlike live spermatozoa, the unstable beating of reactivated spermatozoa caused by high vibration frequencies was not accompanied by an arrest response (Shingyoji *et al.* 1991*a*).

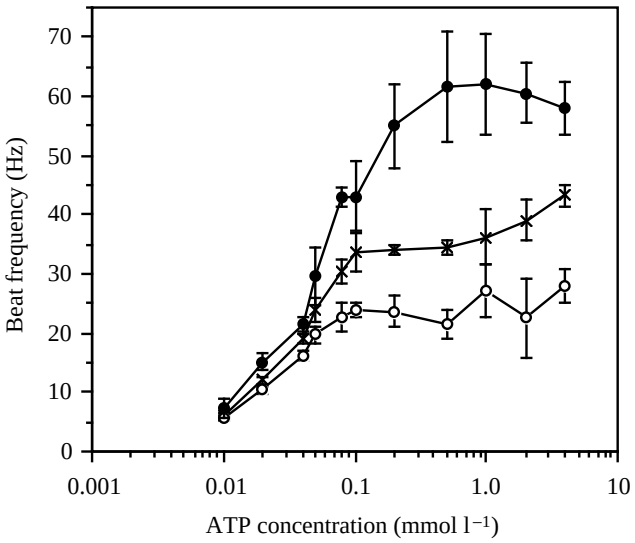

Fig. 2. Range of stable flagellar beating observed at various concentrations of ATP. Crosses, average undriven beat frequency of all the spermatozoa analyzed at the ATP concentration. Filled circles, average maximum frequency capable of maintaining stable beating synchronized to pipette vibration. Open circles, average minimum frequency capable of maintaining stable beating synchronized to pipette vibration. Bars show standard deviations. Number of spermatozoa used were 4–11 for each ATP concentration.

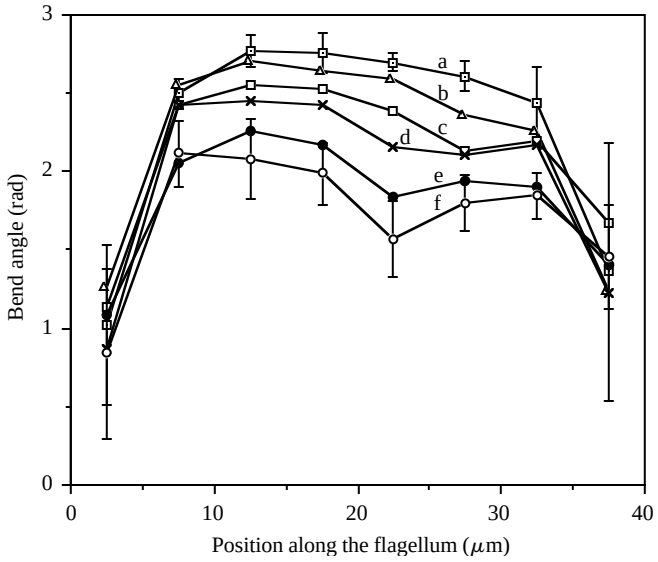

Fig. 3. Changes in bend angles (average bend angles, obtained as averages of principal and reverse bend angles) at various vibration frequencies in flagella reactivated at 2 mmol l^{-1} ATP. Frequencies were: a, 30 Hz; b, 35 Hz; c, 40 Hz; d, 45 Hz; e, 50 Hz; f, 55 Hz. Bars show standard deviations, N=3.

Characteristics of the bending waves during stable beating

Bend angle

Changing the vibration frequency had only a limited effect on the angle of propagated bends in the mid-region of the flagellum. Fig. 3 shows average bend angles (i.e. average of principal and reverse bend angles) along the length of spermatozoa reactivated at 2 mmol l^{-1} ATP while being vibrated at various frequencies. The average undriven beat frequency at 2 mmol l^{-1} ATP was 39.1±3.3 Hz (N=6). The bend angle decreased as vibration frequency increased. The angles of fully developed bends initiated at a frequency of less than 40 Hz remained almost constant in both proximal and mid-distal regions. At vibration frequencies above 40 Hz, the angles decreased gradually as the bends propagated towards the mid-distal region. This decrease in angle resembles that observed in live spermatozoa under imposed vibration (Shingyoji *et al.* 1991*a*).

Fig. 4 shows the effect of frequency on the average bend angle observed in the proximal region, 5–17 μm from the base (open circles), and in the mid-distal region, 25–30 μm from the base (filled circles), of flagella reactivated at various ATP concentrations. At any ATP concentration, the bend angles in the proximal region were equal to or larger than the bend angles in the mid-distal region. At relatively low concentrations of ATP (10, 20 and 50 μmol l^{-1}), the vibration frequency appeared to have little effect on the bend angle, at least up to the frequency 1.25–1.45 times the natural beat frequency. At higher ATP concentrations (100 μmol l^{-1} and 2 mmol l^{-1}), in contrast, the bend angle tended to decrease gradually as the vibration frequency was raised above the undriven beat frequency. The bend angles of the mid-distal regions, at 35 Hz in 100 μmol l^{-1} ATP and at 40 Hz in 2 mmol l^{-1} ATP, were significantly different from those at 45 Hz in 100 μmol l^{-1} and at 50 Hz in 2 mmol l^{-1} ATP (P<0.02 and P<0.05, t-test), respectively.

There were no significant differences between the behaviour of live spermatozoa and reactivated spermatozoa at high ATP concentrations during lateral vibration. Varying the frequency caused a marked change in the wavelength of the flagellar waves (data not shown), in addition to the bend angle, in live spermatozoa, which is similar to the changes seen in *H. pulcherrimus* spermatozoa (see Figs 7 and 8 of Shingyoji *et al.* 1991*a*). Lateral vibration at frequencies higher than the natural beat frequency reduced the wavelength, whereas vibration at lower frequencies increased it by as much as 50 %. Similar changes in wavelength were observed in reactivated spermatozoa at high and low ATP concentrations (data not shown).

Sliding velocity

The effect of vibration frequency on the sliding velocity calculated for several ATP concentrations is shown in Fig. 5. At each vibration frequency, the sliding velocity in the proximal region (5–17 μm from the base) of the flagellum was always larger than that in the mid-distal region (25–30 μm from the base). By and large, the sliding velocity in the proximal region increased with the frequency (Fig. 5A). In contrast, in the mid-distal region (Fig. 5B) the sliding velocity increased with the frequency only up to about 40 Hz, levelling

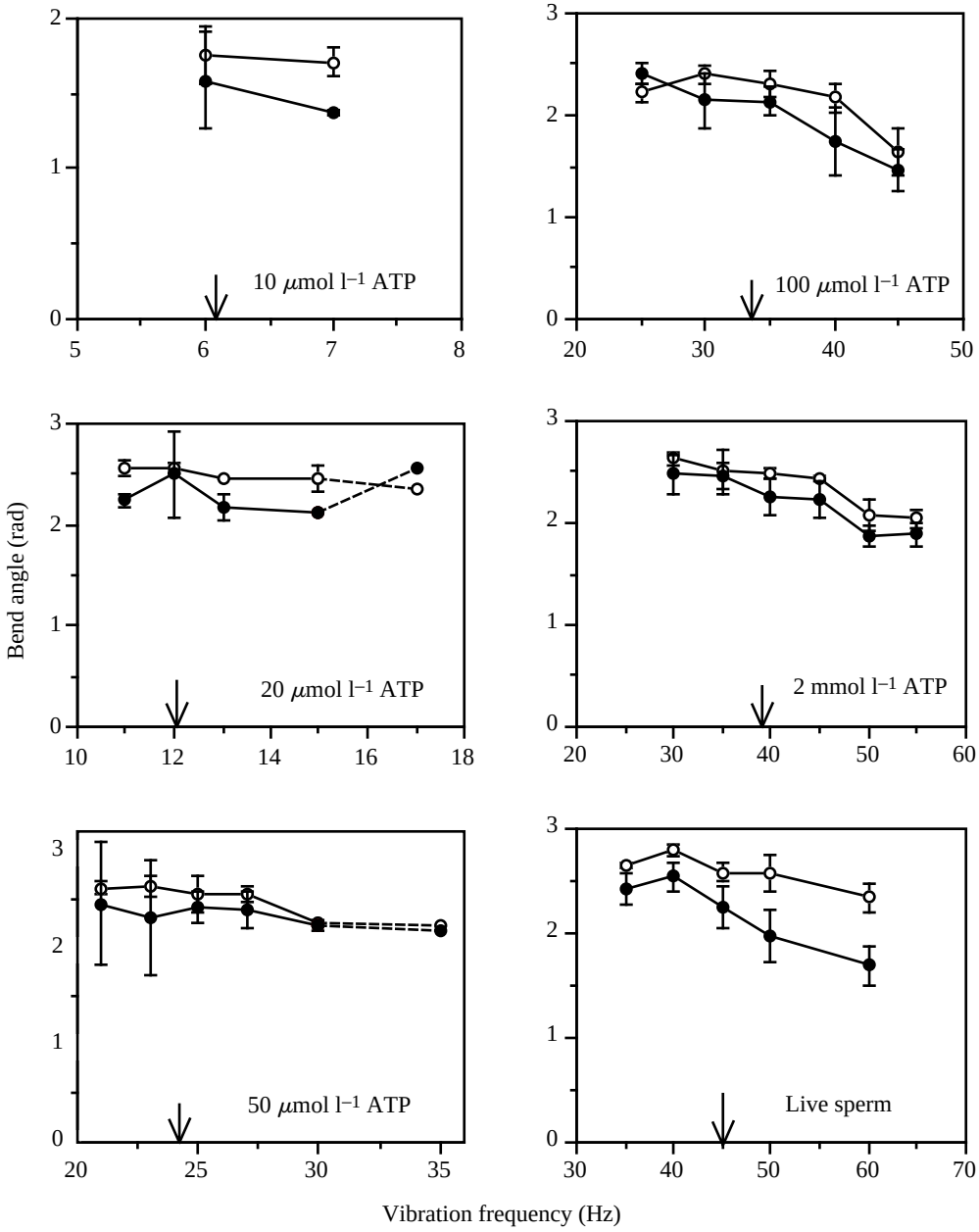

Fig. 4. Changes in average bend angles at various vibration frequencies in flagella reactivated at various ATP concentrations and in live spermatozoa. Open circles, angles in the proximal region (5–17 μm from base); filled circles, angles in the mid-distal region (25–30 μm from base). Bars show standard deviations. The mean undriven beat frequency of the reactivated flagella at each concentration of ATP is indicated by an arrow. Three spermatozoa were analyzed for each frequency except in 10 μmol l^{-1} ATP (two spermatozoa), at 17 Hz in 20 μmol l^{-1} ATP (one spermatozoon) and at 35 Hz in 50 μmol l^{-1} ATP (one spermatozoon).

Table 1. *Apparent sliding velocities in proximal and mid-distal regions of live and reactivated flagella at vibration frequencies above natural (or undriven) beat frequency*

[ATP] (mmol l^{-1})	Frequency range (Hz)	Natural frequency (Hz)	Sliding velocity (rad s^{-1})		*N*
			Proximal*	Mid-distal†	
0.1	35–45	33±3	160±13	140±9	5
2.0	45–55	39±3	217±8	198±10	4
Live sperm	40–60	45±2	254±28	201±3	3

Vibration frequency close to and above the natural or undriven beat frequency. (Sliding velocities were calculated by using the stably beating flagella in this range.)

*Proximal region: 5–17 μm from the flagellar base.

†Mid-distal region: 25–30 μm from the flagellar base.

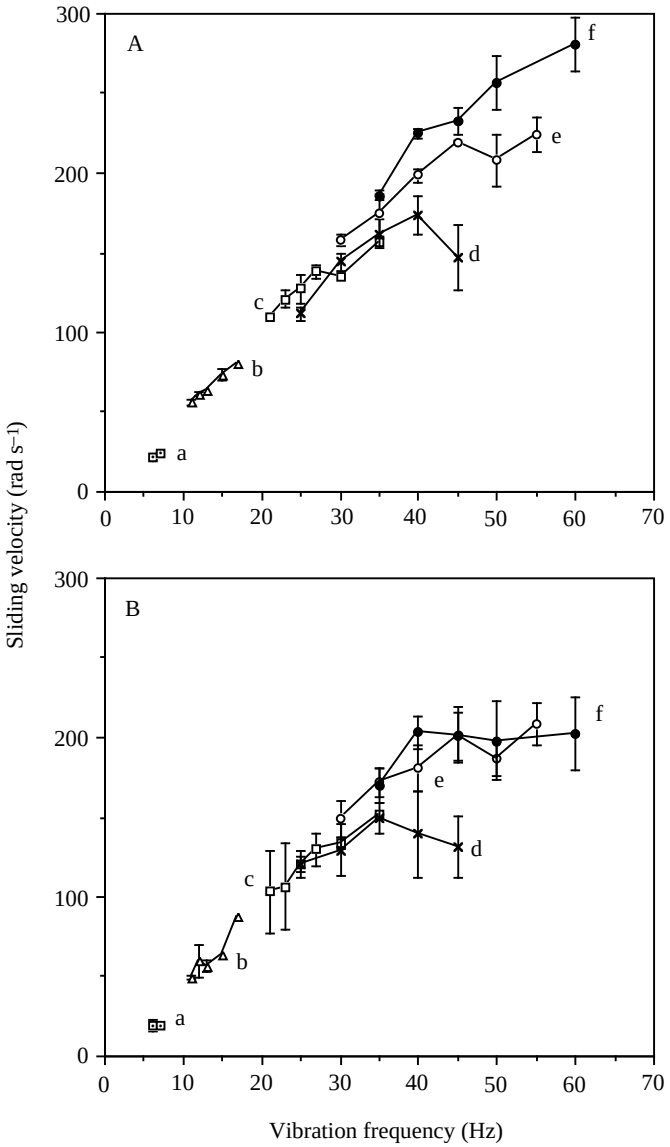

Fig. 5. Effect of vibration frequency on the apparent sliding velocity. (A) Sliding velocity in the proximal region (5–17 μm from base). (B) Sliding velocity in the mid-distal region (25–30 μm from base). a–e, reactivated spermatozoa; f, live spermatozoa. a, 10 μmol l^{-1} ATP; b, 20 μmol l^{-1} ATP; c, 50 μmol l^{-1} ATP; d, 100 μmol l^{-1} ATP; e, 2 mmol l^{-1} ATP. Bars show standard deviations, N=2 (a), 3 (b–f).

off at higher frequencies. At 2 mmol l^{-1} ATP, the mean sliding velocity in the mid-distal region did not apparently change at frequencies above the undriven beat frequency (45, 50 and 55 Hz). The maximal sliding velocity was 198 rad s^{-1}, which was almost the same as the average sliding velocity in the mid-distal region of live spermatozoa (201 rad s^{-1}) (Table 1). At 100 μmol l^{-1} ATP, the average sliding velocity was also constant above the undriven beat frequency, the average value at the vibration frequency of 35–45 Hz being 140 rad s^{-1} (Table 1). We analyzed the data obtained with ATP concentrations of 200 μmol l^{-1}, 500 μmol l^{-1}, 1 mmol l^{-1} and 4 mmol l^{-1}, using one spermatozoon for each concentration. The results, although very tentative because of the small number of observations, indicated that the sliding velocity at 200 μmol l^{-1} ATP was similar to that at 100 μmol l^{-1} ATP, whereas the sliding velocities at higher ATP concentrations were similar to that at 2 mmol l^{-1} ATP (data not shown). The existence of a maximal value of the sliding velocity at higher ATP concentrations was more marked in the mid-distal region of the flagellum than in the proximal region, although maximal values were also obtained in the proximal region of the flagellum.

Table 1 summarizes the average maximal sliding velocities in the proximal and the mid-distal regions of live spermatozoa and spermatozoa reactivated at 100 μmol l^{-1} and 2 mmol l^{-1} ATP. It confirms that at frequencies higher than the undriven beat frequency (e.g. at 50 Hz in 2 mmol l^{-1} ATP) the sliding velocity determined at points 25–30 μm from the flagellar base was not significantly different (P>0.5, t-test) from that observed at frequencies close to the undriven one (e.g. at 40 Hz in 2 mmol l^{-1} ATP).

Fig. 5 also shows that, at 100 μmol l^{-1} and 2 mmol l^{-1} ATP, vibration at frequencies lower than the undriven beat frequency reduces the sliding velocity in both the proximal and mid-distal regions of the flagellum (Fig. 5, lines d and e). When the ATP concentration was lower than 100 μmol l^{-1}, the sliding velocity in both the proximal and mid-distal regions of the flagellum decreased with the decrease of vibration frequency (Fig. 5, lines a–c). At a vibration frequency of about 30 Hz, the sliding velocities at 50 μmol l^{-1}, 100 μmol l^{-1} and 2 mmol l^{-1} ATP were close to 150 rad s^{-1} in the proximal region and 140 rad s^{-1} in the mid-distal region of the flagellum. Fig. 6 shows tracings of these reactivated flagella during vibration at about 30 Hz. Even at different ATP concentrations, the stably beating flagella showed similar waveforms and sliding velocities.

Discussion

General effects of imposed head vibration on reactivated flagella

The present study shows that reactivated sea urchin spermatozoa, like the live spermatozoa studied previously, can be forced to synchronize their flagellar beating with lateral vibration applied to the sperm head over a wide range of frequencies. During lateral vibration, the beat frequency coincides with the imposed vibration frequency along the entire length of the flagellum so that every part of the flagellum beats in a coordinated waveform. The basic characteristics of the observed synchronizing effects in the reactivated flagella do not differ from those observed in live spermatozoa (Shingyoji *et al.* 1991*a*).

The flagellar beat frequency is known to be determined by ATP concentration. However, the observation that the beat frequency at a given ATP concentration can also be changed over a substantial range by imposing lateral vibration on the sperm head indicates that the coupling between the beat

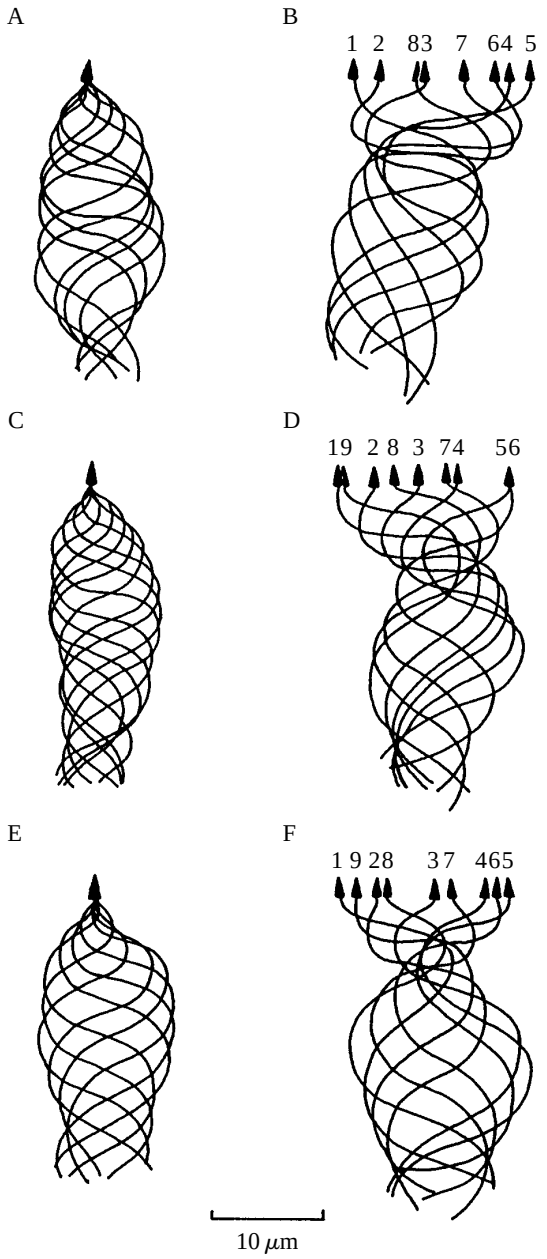

Fig. 6. Tracings of reactivated flagella beating without vibration (A,C,E) and during vibration at about 30 Hz (B,D,F). (A) Reactivated at 2 mmol l^{-1}; beat frequency (undriven beat frequency), 40 Hz. (B) Reactivated at 2 mmol l^{-1}; vibration frequency, 30 Hz. (C) Reactivated at 100 μmol l^{-1}; beat frequency, 31 Hz. (D) Reactivated at 100 μmol l^{-1}; vibration frequency, 30 Hz. (E) Reactivated at 50 μmol l^{-1}; beat frequency, 26 Hz. (F) Reactivated at 50 μmol l^{-1}; vibration frequency, 28 Hz.

frequency and ATP concentration is less tight than has been generally assumed.

The range of vibration frequency that can maintain stable beating has been found to depend on the ATP concentration. The range is wide (20–70 Hz) at ATP concentrations higher than 100 μmol l^{-1}, but becomes narrower at lower ATP concentrations, decreasing gradually with decreasing ATP concentration. The inability to alter vibration frequency and to maintain a stable beat frequency at low ATP concentration may result from the velocity of the imposed movement being too low to strain the basal regions of the flagellum sufficiently. In order to maintain stable beating, sliding between microtubules would have to be coordinated throughout the axoneme. Our results suggest that the mechanism responsible for coordinated sliding may be regulated by ATP concentration.

Sliding velocity

We showed previously that the effects of head vibration on the apparent velocity of microtubule sliding are different in the proximal and the distal regions of the live sperm flagellum (Shingyoji *et al.* 1991*a*). Such positional differences were less pronounced in reactivated flagella, but the sliding velocity was always greater in the proximal region than in the mid-distal region of the flagellum. This is consistent with the hypothesis that the apparently faster sliding in the proximal region might possibly be a result of passive sliding or elastic distortion of the microtubules due to the direct mechanical effect of the vibration (Eshel and Gibbons, 1989).

At ATP concentrations lower than 100 μmol l^{-1}, the apparent sliding velocity changes monotonically with the vibration frequency. This indicates that the ATP concentration is not the primary factor determining the sliding velocity in this range. At ATP concentrations higher than 100 μmol l^{-1}, the apparent sliding velocity showed biphasic changes with the change of vibration frequency. Above the undriven beat frequency it remained constant, suggesting that the concentration of ATP is the primary factor determining the microtubule sliding velocity which, in turn, determines the beat frequency.

Studies of the effects of magnesium concentration and those of elastase on flagellar motility showed an increase in beat frequency with a decrease in bend angle, or *vice versa*; as a result, a constant sliding velocity is maintained (Okuno and Brokaw, 1979; Brokaw, 1980). The present data are the first report indicating a possible change in sliding velocity when beat frequency is altered. Okuno and Brokaw (1979) reported that there was no change in bend angle of attached spermatozoa as ATP concentration was varied. This is consistent with our data showing that bend angles of the spermatozoa vibrated at natural beat frequencies in ATP concentrations higher than 20 μmol l^{-1} were almost constant.

Both the range of vibration frequency capable of maintaining stable beating and the effect of vibration frequency on the apparent sliding velocity showed differences, depending upon whether the ATP concentration was above or below approximately 100 μmol l^{-1}. This raises the question of whether this concentration of ATP has a special significance. It is interesting that certain other properties of dynein, as well as the characteristics of ATP-driven sliding disintegration of axonemes, show a similar difference with ATP concentration. Toyoshima and Miki-Noumura (1990) reported that, in motility assay experiments *in vitro*, the A-band protein of 13 S dynein obtained from sea urchin sperm flagella can support

translocation of microtubules over glass only when the concentration of ATP is above 100 μmol l^{-1}, although it can induce dissociation of axonemes at lower concentrations. Tanaka and Miki-Noumura (1988) have shown that the completeness of ATP-induced disintegration of demembranated cilia from *Tetrahymena pyriformis*, as measured by turbidity changes of the axonemal suspension, is proportional to ATP concentration up to a maximum reached at 100 μmol l^{-1} ATP, and that this maximum is maintained at higher concentrations. Kobayashi *et al.* (1990) have found that when demembranated sea urchin sperm flagella are digested with elastase in the presence of ATP, the axonemes do not disintegrate completely into individual microtubules. Instead, only one or two episodes of sliding take place, suggesting that active sliding can occur only between one or two pairs of the nine doublets. Unpublished observations from our laboratory show that this pattern of disintegration occurs only when the concentration of ATP is above 50 μmol l^{-1} and that sliding occurs between more than three doublet pairs at lower ATP concentrations (C. Shingyoji, T. Kobayashi and K. Takahashi, in preparation). We do not know whether there is a common explanation for all these results, but it is possible that the ATP-sensitive activity of the dynein arm itself or the mechanism regulating active sliding to produce coordinated sliding change qualitatively at an ATP concentration of about 100 μmol l^{-1}.

Regulation by ATP concentration

The beat frequency of reactivated flagella is related to the MgATP concentration by quasi Michaelis–Menten kinetics (Brokaw, 1967, 1975; Gibbons and Gibbons, 1972). The sliding velocity between microtubules during ATP-induced disintegration shows a similar dependence on MgATP concentration (Takahashi *et al.* 1982). Kamimura and Kamiya (1992) have found nanometre-scale high-frequency oscillations (about 300 Hz) along the longitudinal axis of non-beating flagellar axoneme that change frequency with ATP concentration. These findings support the widely accepted view that MgATP is the main factor in determining the rate of flagellar motility. However, we do not yet know whether the flagellar beat frequency is governed by a flagellar oscillator that is directly regulated by the MgATP concentration or whether the beat frequency is determined indirectly by active sliding velocities controlled by MgATP concentration.

In our experimental conditions, we changed ATP concentration without changing magnesium concentration; as a result, free Mg^{2+} concentration ranged from 1.30 to 2.16 mmol l^{-1}. We suggest that the change in the parameters of movement due to this range of [Mg^{2+}] does not significantly alter our conclusions (Okuno and Brokaw, 1979).

The idea that the beat frequency is determined indirectly as a result of an effect of ATP concentration on the maximum sliding velocity is attractive because the sliding velocity seems to be a fundamental variable of an active sliding system. If this is the case, some indications of an inverse relationship between beat frequency and bend angle might be expected, especially at low ATP concentrations where sliding velocity, rather than a balance of moments, might be the major determinant of beat frequency (Brokaw, 1975). In our present study, neither increasing nor decreasing the beat frequency at low ATP concentrations changed the bend angle but both changed the apparent sliding velocity. This indicates that the sliding velocity does not depend solely upon ATP concentration, but could also be controlled by an oscillatory mechanism closely associated with the mechanism initiating new bends at the flagellar base.

The ability of short regions of a flagellum to oscillate independently has been demonstrated by experiments in which demembranated flagella are locally reactivated with ATP (Brokaw and Gibbons, 1973; Shingyoji *et al.* 1977). A local bend formed in a short region of a flagellum can be propagated along the flagellum (Okuno and Hiramoto, 1976; Shingyoji *et al.* 1977). These results indicate that initiation of bending waves is a process independent of bend propagation. In order to produce a coordinated oscillatory movement, sliding along each doublet pair must be coordinated. In the present study, the flagellum was forced to synchronize its beat with the imposed vibration so that the beat frequency could be changed over a wide range at a given ATP concentration. This implies that not only the initiation but also the propagation of bending waves can be modulated by the imposed vibration. ATP concentration, therefore, affects the velocity of active sliding between each doublet pair, as well as the oscillatory mechanism associated with bend initiation and the oscillatory mechanism responsible for coordinated sliding along the flagellum. It is possible that the beat frequency is determined by the combined effect of these three processes. If this were the case, the relationship between frequency and MgATP concentration would be more complicated than that expressed by simple Michaelis–Menten kinetics.

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