CELL STRUCTURE AND FUNCTION 32: 17–27 (2007)
© 2007 by Japan Society for Cell Biology

Induction of Beating by Imposed Bending or Mechanical Pulse in Demembranated, Motionless Sea Urchin Sperm Flagella at Very Low ATP Concentrations

Rina Ishikawa[1] and Chikako Shingyoji[1]*

[1]*Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Tokyo, 113-0033, Japan*

ABSTRACT. **A basic feature of the movement of eukaryotic flagella is oscillation. Although flagellar oscillation is thought to be regulated by a self-regulatory feedback system including the mechanical signal of bending itself, the mechanism regulating the dynein motile activity to produce oscillation is not well understood. To elucidate the mechanism, we developed a new experimental system which allowed us to analyze the conditions necessary for the induction of oscillation. When a mechanical signal of bending or a pulse was applied by micromanipulation to a demembranated motionless sea urchin sperm flagellar axoneme at very low ATP concentrations (1–3 μM), a localized pair of bends was induced. The bend formation was often followed by further responses including propagation of the distal bend of paired bends, growth and propagation of the paired bends, and cyclical beating. The beating was induced at 2.0 μM or higher concentrations of ATP, but appeared even at 1.5 μM ATP if a few μM of ADP was also present. When the proximal half of a flagellum was attached to a microneedle, beating could not be induced in the distal free region at 2 μM ATP. These results suggest that mechanical signal is involved in the mechanism regulating the motile activity of dynein to produce oscillation. Our results also showed that the presence of a small amount of ADP and the axial difference along the flagellum are factors essential for the induction of flagellar oscillation.**

Key words: bend initiation/dynein/microtubule sliding/mechanical signal/ADP

Introduction

Oscillation is a prominent feature of eukaryotic flagella. In sea urchin sperm, planar bending waves are formed at the base of the flagellum and propagate towards the distal end. The bending is caused by ATP-dependent, dynein-driven microtubule sliding (Summers and Gibbons, 1971; Shingyoji *et al*., 1977; Brokaw, 1991). Although all the dynein arms on the doublet microtubules of a flagellum are capable of moving the adjacent doublets (Sale and Satir, 1976), the speed and extent of microtubule sliding vary during a beat cycle according to the position in the flagellum and the phase of the bending cycle. In demembranated sea urchin sperm flagella, bending waves can be generated by ATP (Gibbons and Gibbons, 1972; Brokaw, 1975), and local application of ATP to any region of the demembranated flagellum induces a localized pair of bends or localized beating (Shingyoji *et al*., 1977). The local reactivation experiments corroborated the microtubule sliding model of ciliary and flagellar movement and identified some conditions for bend formation (Shingyoji *et al*., 1977; Takahashi *et al*., 1982), but are by themselves of limited use for further analysis of the mechanism of flagellar oscillation. In the sea urchin sperm flagella, bend formation in one direction is usually followed by bending in the opposite direction, and this cyclical formation of bends is always coupled with growth and propagation of the bends. These coupling phenomena make it difficult to analyze the factors governing the induction of flagellar oscillation. To overcome this difficulty a new method to control the timing of the induction of oscillatory movement was deemed necessary.

Previous studies have demonstrated that certain aspects of flagellar movement, e.g. beat frequency and wave initiation, can be modified by mechanical manipulation of the flagellum (Okuno and Hiramoto, 1976; Gibbons *et al*., 1987; Shingyoji *et al*., 1991; Shingyoji *et al*., 1995). It has also been shown that inactive dynein arms in the axonemes are activated by bending the doublet microtubules (Hayashibe *et al*., 1997; Morita and Shingyoji, 2004). The activity of

*To whom correspondence should be addressed: Chikako Shingyoji, Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Tokyo, 113-0033, Japan.
Tel: +81–3–5841–4065, Fax: +81–3–5841–4385
E-mail: chikako@biol.s.u-tokyo.ac.jp

the dynein arms thus appears to be modulated, in some cases enhanced, by the mechanical environment or mechanical "signals". It seems probable that bending of the doublet microtubules constitutes an important factor in the self-regulatory feedback system underlying the flagellar oscillation (Morita and Shingyoji, 2004).

To analyze the factors governing flagellar oscillation, we studied the effect of bending demembranated, motionless flagella by micromanipulation in the presence of ATP at concentrations near or below the threshold for inducing spontaneous beating. By this method we were able not only to induce but also to control the timing of flagellar oscillation. Our results showed that the motile activity of dynein can be increased by the mechanical effect, thereby inducing oscillation even at very low ATP concentrations.

It has been demonstrated that ADP is involved in the regulation of flagellar motility and dynein activity (Kinoshita *et al*., 1995; Omoto *et al*., 1996; Yagi, 2000; Kikushima *et al*., 2004). Our study indicated that the presence of a very low concentration of ADP is essential for continuous beating at very low ATP concentrations. The difference in the species of dynein and/or the regulatory mechanism of the motile activity of dynein between the proximal and the more distal regions of the flagellum may also be an important factor determining the induction of flagellar oscillation.

Materials and Methods

Demembranation and reactivation of sperm flagella

Spermatozoa of the sea urchin, *Pseudocentrotus depressus,* were demembranated according to the previously described methods (Yoshimura and Shingyoji, 1999). Demembranation was stopped by adding reactivating solution without ATP. Demembranated sperm that showed 80% or higher reactivation rate at 200 μM ATP were used for experiments. All experiments at low ATP concentrations were performed within 10 min after the ATP addition. For experiments with ADP, ADP was added 15 sec before the addition of ATP. To reduce the concentration of ADP to as low as <0.1 μM (Shiroguchi and Toyoshima, 2001), we used an ATP regeneration system. For this experiment, we treated the demembranated sperm with 20 mM creatine phosphate and 0.2 mg/ml creatine kinase for 3 min on ice before the addition of ATP. For each condition we obtained the data from three to eleven separate experiments, in each of which one to three sperm flagella were usually studied. All experiments were carried out at room temperature (20–23°C).

Mechanical manipulation

A glass microneedle with the tip coated with 0.1% poly-L-lysine (P-1264, Sigma, U.S.A.) was mounted on a micromanipulator (WR-88, Narishige, Tokyo, Japan) and the head of a demembranated motionless sperm was attached to the tip. To bend the flagellar axoneme, the distal end of the axoneme was attached to another glass microneedle, which was then moved with respect to the head. To apply a mechanical pulse through the glass microneedle holding the sperm head, the manipulator was touched lightly by hand.

Observation, recording and analysis of flagellar movement

Flagellar responses to bending or a mechanical pulse were observed under an inverted microscope (TMD, Nikon, Tokyo, Japan) equipped with a dark-field condenser (Nikon, Tokyo, Japan) and a ×40 objective lens and recorded on video tape by using a CCD camera (Watec Neptune 100, Watec Co., Japan) with an image intensifier (DII-2050, Nakanishi Image Lab Inc., Tokyo, Japan) and a VHS videocassette recorder. The beat frequencies of flagella were measured by using a stroboscopic light source (Model 271C, Chadwick-Helmuth Co., U.S.A.). Recorded images of the flagella and the glass microneedles were traced from the monitor screen onto a transparent sheet by hand.

Results

Reactivation of flagella at very low ATP concentrations

Fig. 1A shows the average reactivation rates, i.e. the percentage of motile sperm, at various ATP concentrations. As we did not use an ATP regeneration system, the sperm

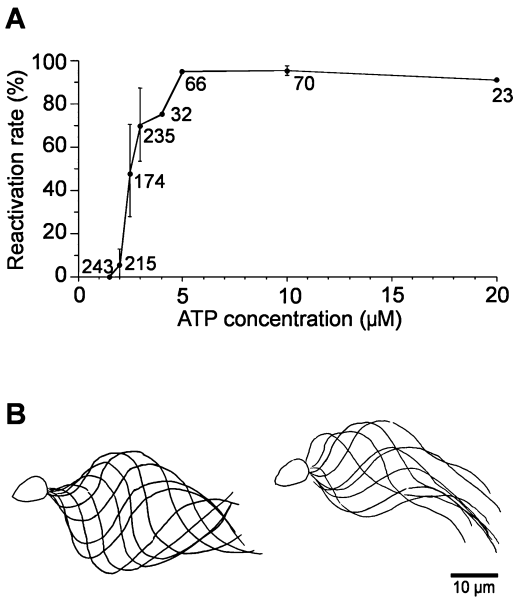


Fig. 1. Reactivation of sperm flagella at low ATP concentrations. (A) Average reactivation rates of demembranated flagella at 1.5–20 μM ATP. Each plot with a bar indicates mean±s.d. Numbers beside the plots show the total numbers of sperm observed. (B) Tracings of waveforms showing oscillatory beating with complete (left) and incomplete (right) propagation at 3.0 μM ATP. The sperm heads were attached to the glass surface.

density was kept as low as possible so that the flagellar beat frequency at 3.0 μM ATP stayed almost constant for more than 10 min. Below 5.0 μM ATP, the rate of reactivation decreased with the ATP concentration. The wave propagation was also influenced by ATP. At 3.0 μM ATP, most of the beating sperm showed normal oscillatory waves with complete propagation along the length of the flagellum (Fig. 1B, left), while in others the bending waves were propagated only in the proximal and middle regions (Fig. 1B, right). The number of flagella showing incomplete propagation increased as the ATP concentration decreased. At 2.0 μM ATP, 5.8% of sperm were motile and all sperm became motionless at 1.5 μM ATP. The motionless flagella at 1.0–3.0 μM ATP were either straight or showed waves of very small amplitude. In contrast, the flagella in the absence of ATP had a clear bend or S-shaped bends showing so-called rigor waves (Gibbons and Gibbons, 1974).

Flagellar responses to imposed bending

In the first series of experiments, we bent the flagellum of a motionless sperm by moving the distal end of the flagellum with respect to the head in the presence of 1.0–4.0 μM ATP. This induced a bend or bends in the flagellum (Fig. 2A and 2B). When the whole length of the flagellum was bent in one direction, a single bend was induced at the base of the flagellum (Fig. 2A). Such a bend, which was induced in 16 flagella, did not show further development. In contrast,

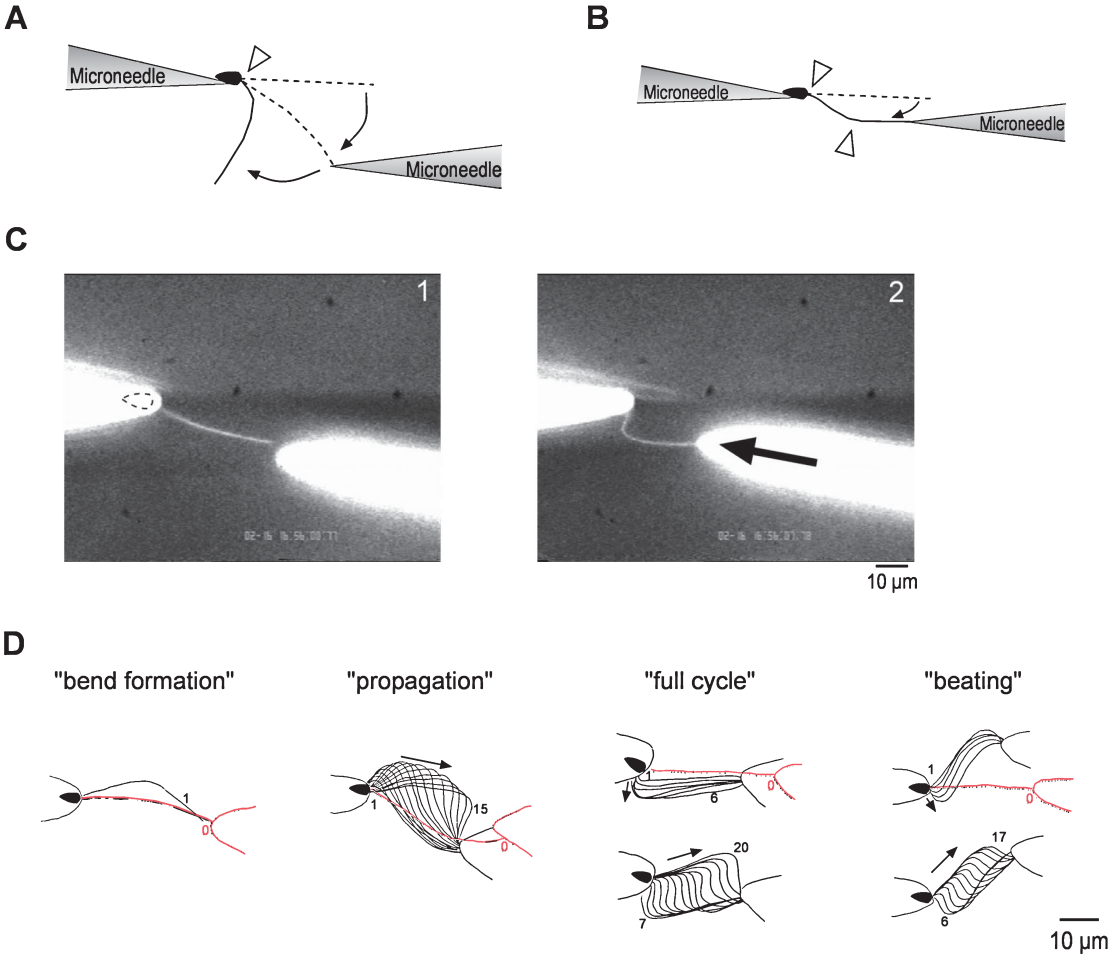


Fig. 2. Effects of imposed bending on motionless flagella. (A) and (B) Schematic diagrams showing two ways of inducing bending in a motionless sperm whose head was held by the left microneedle. Bending the whole flagellum in one direction with the right microneedle induced a bend at the base of the flagellum (A), while pushing the distal end of the flagellum towards the head induced a pair of bends (indicated with open arrowheads) (B). (C) Video images showing the motionless flagellum at 2.5 μM ATP before (1) and during (2) mechanical deformation imposed as shown in B. The sperm head (indicated with a dotted line in 1) was held by the left microneedle (images overlapped). The tip of the flagellum was caught by the right microneedle. (D) Four types of flagellar response observed under imposed bending shown by sequential tracings from video images. The position of the flagellum (numbered with zero) and a glass microneedle before each mechanical deformation are indicated in red. Numbers indicate the sequence of tracings. In "beating", changes of waveforms are shown only for the first of two or more cycles. Arrows indicate the direction of bend propagation.

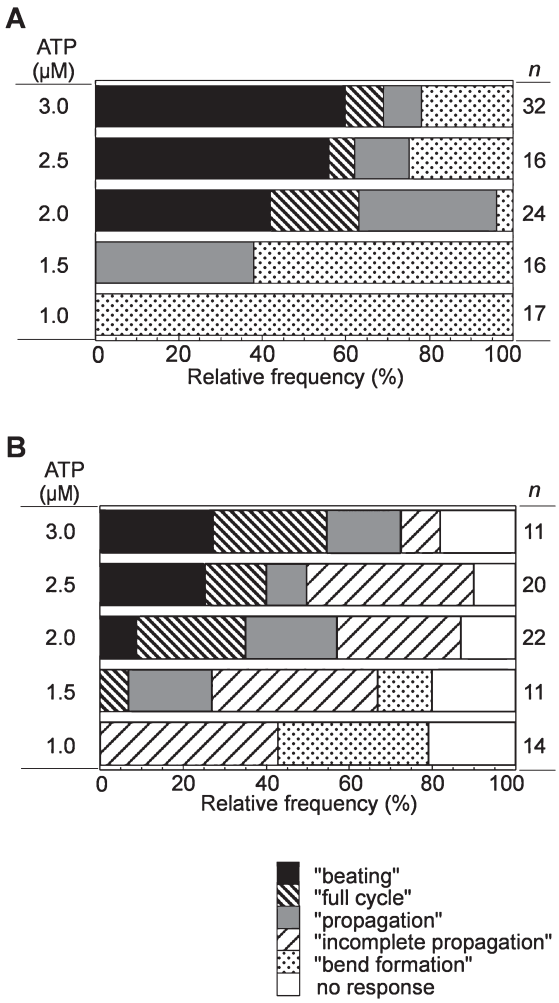


Fig. 3. Effects of mechanical manipulation on the frequency of occurrence of flagellar responses at low concentrations of ATP (1.0–3.0 μM). (A) Mechanical deformation imposed on motionless flagella induced four types of response: formation of a pair of bends, propagation of the distal bend of a pair of bends, growth and propagation of the proximal bend following the propagation of the distal bend, and beating for two or more cycles, which were named as "bend formation", "propagation", "full cycle, and "beating", respectively. (B) A mechanical pulse applied to motionless flagella induced five types of response, which included propagation of the distal bend of a pair of bends for a part of the flagellum ("incomplete propagation") in addition to the four types shown in (A).

when the flagellum was deformed so as to induce a pair of bends along the flagellum (Fig. 2B and 2C), the induced bends usually showed further development (Fig. 2D).

Induction of a pair of bends (which we call "bend formation") was often followed either by propagation of the distal bend of the pair ("propagation"), or by growth and propagation of the proximal bend to accompany those of the distal bend (a "full cycle"), or by beating for two or more cycles ("beating") (Supplemental Video 1), in the apparent order of development. All of the four types of response were induced at 2.0–3.0 μM ATP, but "beating" and "full cycle" were not induced at lower concentrations (Fig. 3A).

Table I. BEAT FREQUENCIES OF SPONTANEOUS AND INDUCED BEATING

Nucleotide concentration [μM]		Beat frequency [Hz][a)]	
ATP	ADP	Spontaneous beating	Induced beating
3.0	0	0.4±0.06 (69)*	0.3±0.1 (12)*
2.5	0	0.2±0.05 (16)**	0.1±0.08 (11)**
2.0	0	0.1±0.07 (17)	0.09±0.03 (10)[§1]
1.5	0	0	Not induced
1.5	1.5	0	0.04±0.01 (7)[§2]
1.5	3.0	0	0.04±0.03 (4)[§3]
1.5	10.0	0	0.06±0.03 (5)

[a)] Mean±s.d. (*n*).
*, **, § (between [§1] and the group of [§2] and [§3]), The differences are statistically significant (Mann-Whitney U test, $P<0.05$).

The flagellar response was observed only under imposed bending; removal of the mechanical constraint by detaching the microneedle from the tip of the flagellum terminated the responses at any moment, restoring the almost straight form of the flagellum (Supplemental Video 2). Induction of the four types of flagellar response by mechanical deformation was reproducible: applying the deformation again to the same flagella (n=27), induced the same response as the first one in 37% of them, while in 56% of them the responses were of the type a step more developed; for example, a "full cycle" in the first and "beating" in the second trial. The threshold concentration of ATP (2.0 μM) for induction of "beating" did not change between the first and the second trials. A microneedle, which was attached to the distal end of the flagellum, was moved at 3–41 μm/s for a distance of 2.2–34.9 μm, but the differences in these parameters did not affect the rates of occurrence of the four types of response.

The beat frequency of the mechanically induced "beating" increased with the ATP concentration like the frequency of spontaneous beating. However, the average beat frequencies of the mechanically induced "beating" at 2.0–3.0 μM ATP were slightly lower than those of the spontaneous beating (Table I): at 2.5 and 3.0 μM ATP the differences were statistically significant (Mann-Whitney U test, $P<0.05$).

Induction of flagellar responses by a mechanical pulse

In the second series of experiments, we used only one microneedle to hold the sperm head, leaving free the distal end of the flagellum, and applied a mechanical pulse by touching the micromanipulator holding the microneedle. In many cases the pulse induced a pair of small bends near the base of the flagellum (Fig. 4A and 4B). In most cases the formation of bends ("bend formation") was followed by one of the five types of flagellar response (Figs. 3B and 4C). Except for the cases in which the distal bend of the pair

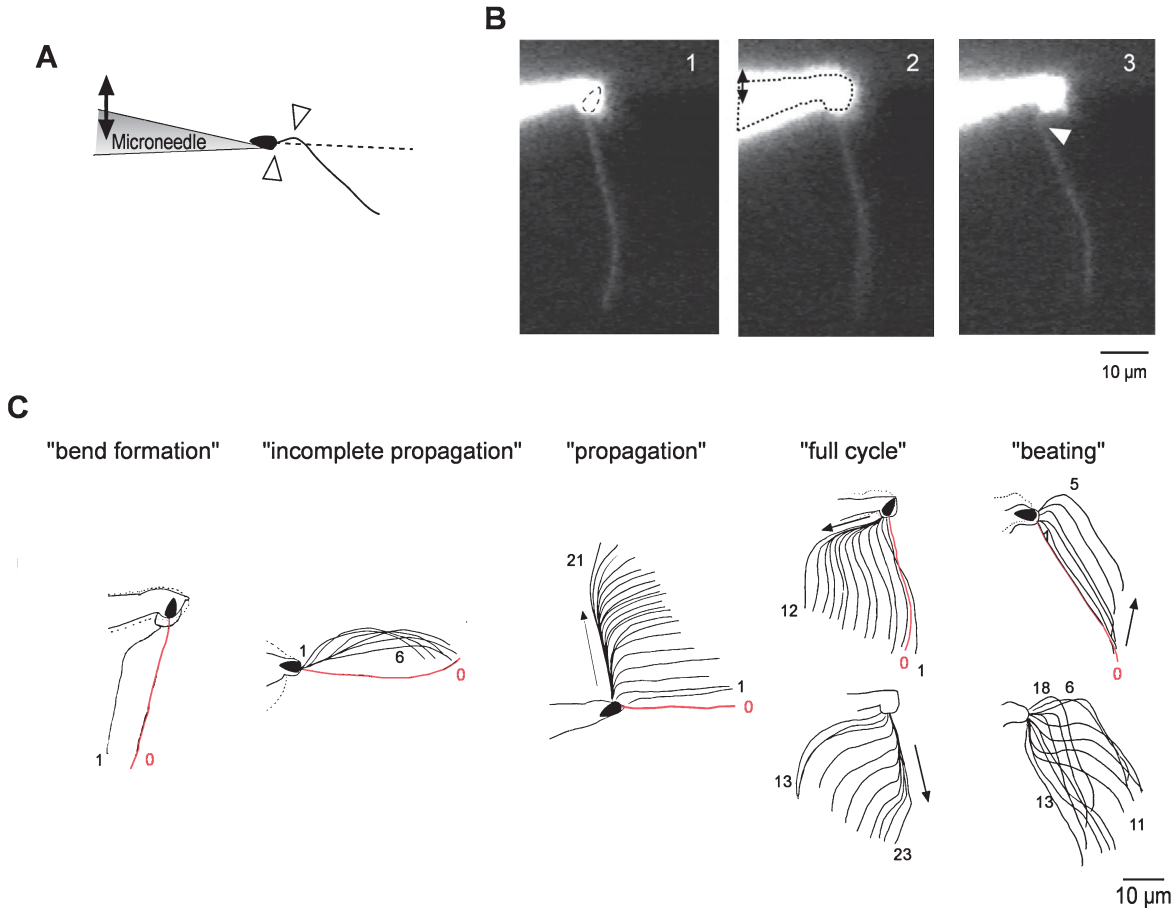


Fig. 4. Effects of a mechanical pulse on motionless flagella. (A) Schematic diagram showing the method of applying a mechanical pulse through the microneedle holding the sperm head. A light tap on the micromanipulator on which the microneedle was mounted induced a pair of small bends in the proximal region of the flagellum (indicated with two open arrowheads). (B) Sequential video images showing a demembranated sperm at 2.5 µM ATP before (1), during (2), and after (3) the application of a mechanical impulse. The distal bend of the pair of bends induced by the mechanical pulse was indicated with a white arrowhead. (C) Sequential tracings from the video images showing five types of flagellar response induced by the mechanical pulse. The position of the flagellum before each mechanical pulse was indicated in red (numbered with zero). The numbers show the sequence of tracings. Arrows indicate the direction of bend propagation.

propagated for a part of the flagellum ("incomplete propagation"), the four types of response were similar to those induced by bending. All five types of flagellar response proceeded in the absence of a further mechanical pulse. This observation supports the idea that the flagellar response induced by the mechanical pulse involved the activity of dynein and was not a passive elastic response of the demembranated sperm.

Fig. 3B summarizes the frequencies of occurrence of the five types of response induced by the mechanical pulse at different ATP concentrations. "Beating" was induced less frequently by a mechanical pulse than by bending (Fig. 3A), but the critical ATP concentration for inducing "beating" was the same in both methods (2.0 µM ATP) (Fig. 3A and 3B). The speed (8–24 µm/s) and the amplitude (1.3–2.5 µm) of the movement of the microneedle in the mechanical pulse did not affect the occurrence of the types of response.

Characteristics of the induced bends

Comparison of the pair of bends before and during their development into further flagellar responses for different types of responses showed that the position and angle of the distal bends were correlated with the types of the flagellar response, regardless of the ATP concentration. Table II summarizes the average position (distance from the base measured along the flagellum) and the average angle of the initially induced distal bends and the average angle of the maximally grown distal bends for each type of flagellar response induced at 1.0–3.0 µM ATP.

In the bending experiments, the more developed responses among the four types (beating>full cycle>propagation>bend formation) were observed when the first pair of bends was induced in a region closer to the flagellar base. The difference in the distance was statistically significant (*1–*4 in

Table II. CHARACTERISTICS OF MECHANICALLY INDUCED BENDS[a)]

Method	Response	Distance of the distal bend from the base [μm]	Angle of the distal bend [rad]		Max. angle of the grown distal bend [rad]
Imposed bending	"beating"	2.9±1.9 (17)*[1]	1.2±0.4 (17)	§[1]	2.0±0.3 (8)**
	"full cycle"	4.4±2.1 (13)*[2]	1.1±0.3 (13)	§[1]	1.7±0.4 (10)**
	"propagation"	6.5±4.5 (14)*[3]	1.3±0.3 (14)	§[1]	1.4±0.4 (14)**
	"bend formation"[b)]	17.6±7.7 (15)*[4]	1.0±0.3 (15)[§1]		1.0±0.3 (15)**
Mechanical pulse	"beating"	3.1±1.9 (7)	1.2±0.4 (7)	§[2]	1.8±0.3 (7)
	"full cycle"	4.9±1.7 (6)	1.1±0.4 (6)	§[2]	1.8±0.2 (6)
	"propagation"	5.4±2.8 (7)	1.3±0.1 (4)	§[2]	1.6±0.2 (4)
	"incomplete propagation"	5.5±1.4 (14)	1.0±0.5 (10)	§[2]	1.0±0.5 (10)
	"bend formation"	5.1±1.4 (3)	0.9±0.2 (3)	§[2]	0.9±0.2 (3)

[a)] Characteristics of distal bends of each pair of bends induced at 1.0–3.0 μM ATP. Mean±s.d. (*n*).
[b)] Analyses were made of bends that remained after removal of mechanical force.
**, The difference between any two of these are statistically significant (Mann-Whitney U test, $P<0.05$).
*[1–*4], The difference between any two of these except between *[2] and *[3] are statistically significant (Mann-Whitney U test, $P<0.05$).
§[1], §[2], The differences between the angles of the responses with propagation (including "beating", "full cycle" and "propagation") and those of the responses without propagation (including "incomplete propagation" and "bend formation") are statistically significant (Mann-Whitney U test, $P<0.05$).

Table II; Mann-Whitney U test, $P<0.05$) between any two of the flagellar responses, except between the "full cycle" and the "propagation". In the mechanical pulse experiments, "beating" was also induced when a pair of bends was formed closer to the base. It is interesting that "beating" and "full cycle" were developed from bends formed at similar positions (3.1 μm and 4.9 μm) in both the bending and the mechanical pulse experiments (Table II).

In both types of experiments, the bend angles of the distal bend did not differ much among the types of flagellar response, but when we compare the angles between the flagellar responses with and without propagation (§[1] and §[2] in Table II) it is clear that the responses with propagation grew from bends with larger angles (Mann-Whitney U test, $P<0.05$). The growth of the distal bends was also correlated with the type of response. In the bending experiments the larger the maximum bend angles, the more developed the type of response became (** in Table II; Mann-Whitney U test, $P<0.05$). More precisely, the threshold angles of the distal bends for the induction of "propagation", "full cycle" and "beating" were about 1.4 rad, 1.7 rad and 2.0 rad, respectively. The proximal bend of the first pair of bends also grew up to 1.8±0.4 rad (n=10) for "full cycle" and 2.2±0.3 rad (n=8) for "beating". There was no correlation between the curvature of the distal bend and the type of response.

Effect of ADP on the flagellar responses

Without ATP, bending the demembranated flagella in the presence of 1–100 μM ADP did not induce further flagellar responses. At 1.5 μM ATP, in the absence of ADP, the mechanically-induced pair of bends developed into "propagation" in 40% of the flagella (Fig. 5), while "full cycle" and "beating" did not appear. In the presence of both ADP and ATP at 1.5 μM, however, "full cycle" and "beating" as well as "propagation" were induced by imposed bending (Fig. 5). The position and the angle of the initial bends induced at 1.5 μM ATP with ADP could not be measured because the bends were not in the plane of focus.

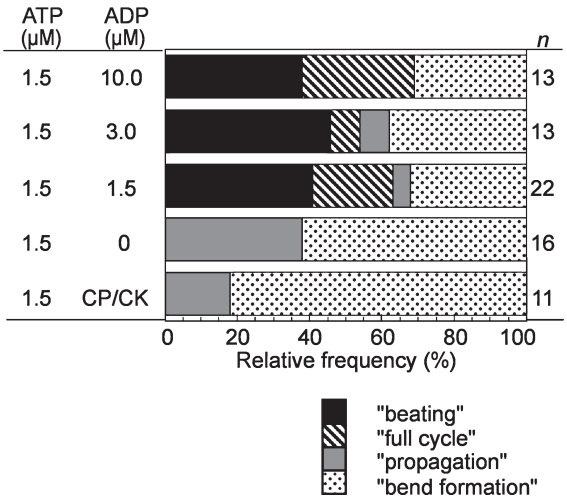


Fig. 5. Effects of the presence of ADP on the flagellar responses induced by imposed bending of motionless flagella at 1.5 μM ATP. In the presence of ADP "beating" and "full cycle" appeared. The concentration of ADP did not affect the rate of occurrence of these responses. CP/CK: in the presence of an ATP regeneration system, creatine phosphate and creatine kinase, instead of ADP.

The frequencies of occurrence of the "full cycle" (20%) and "beating" (40%) were similar to those induced at 2.0

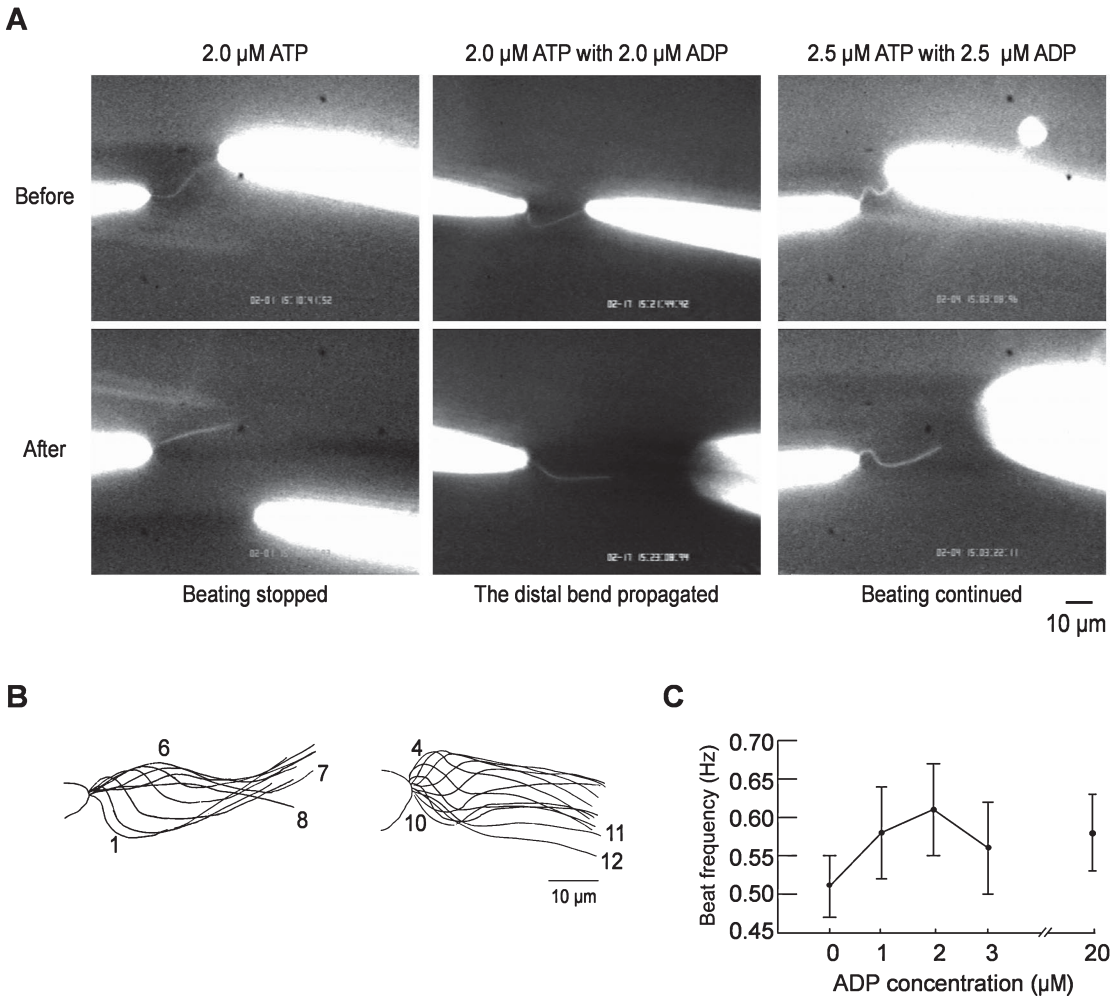


Fig. 6. Increase of the oscillatory ability of flagella by ADP. (A) Video images showing "beating" before and after the removal of mechanical manipulation. By detaching the right microneedle from the flagellum, "beating" stopped at 2.0 μM ATP (left), the last distal bend propagated at 2.0 μM ATP with ADP (center), and beating continued at 2.5 μM ATP with ADP (right). (B) Sequential tracings showing two examples of continuous beating after removal of the microneedle at 2.5 μM ATP with 2.5 μM ADP. The left tracings shown are from the same flagellum as shown in A (right). Numbers indicate the sequence of tracings. (C) Effects of ADP on the average beat frequency of spontaneously beating flagella at 3.0 μM ATP. Each plot indicates mean±s.d. (n=7–11). All average beat frequencies in the presence of ADP are significantly larger than the beat frequency in the absence of ADP (Mann-Whitney U test, P<0.05).

μM ATP without ADP (Fig. 3A). In contrast, at 1.5 μM ATP with 1.5–10 μM ADP the spontaneous beating, which was observed at 2.0 μM ATP, was never observed (Fig. 1A). The beat frequency as well as the frequencies of occurrence of "beating" at 1.5 μM ATP with ADP did not significantly change with the ADP concentration (1.5, 3.0 and 10 μM) (Table I). The average beat frequency at 1.5 μM ATP with 1.5 and 3.0 μM ADP (0.04 Hz) was significantly lower than that of the induced "beating" at 2.0 μM ATP (0.09 Hz) (Table I). These observations indicate that ADP does not function simply as a substrate of adenylate kinase to increase the ATP concentration. The presence of the ATP regeneration system (CP/CK) at 1.5 μM ATP did not change the frequency of occurrence of the responses but decreased the rate of occurrence of "propagation". The results indicate that ADP may influence the motile activity of dynein so as to induce flagellar oscillation.

We found that ADP was also related to the stability of cyclical beating. As mentioned above, removal of the mechanical constraint by detaching the microneedle from the distal end of the flagellum terminated the flagellar response and the flagellum became straight. This was observed at 2.0–4.0 μM ATP. A typical example is shown in Fig. 6A (left) in which the "beating" induced by the imposed bending at 2.0 μM ATP (left top) stopped by detaching the right microneedle from the tip of the flagellum and the bends relaxed (left bottom). However, in the presence of 2.0 μM ADP in addition to ATP (center) the

removal of the microneedle induced propagation of the last bend at more distal region of the flagellum (center bottom) before the stoppage of "beating" (center top). This was observed in one flagellum. We then examined the effect of the presence of both ADP and ATP at 2.5 μM. In three of the seven flagella studied flagella continued beating for 1–5 cycles after the removal of the microneedle (Fig. 6A right and 6B) (Supplemental Video 3).

The beat frequency of the mechanically induced "beating" in the presence of both ADP and ATP at 2.5 μM (0.3–0.4 Hz) was higher than the average beat frequency of the mechanically induced "beating" at 2.5 μM ATP without ADP (0.1 Hz). It seems likely that the continuous beating was achieved by an ADP-dependent increase of the oscillatory ability of the flagella. The beat frequency at 2.5 μM ATP in the presence of ADP (0.3–0.4 Hz) is near the maximum beat frequency of spontaneous beating without ADP (0.14–0.37 Hz). A similar increase of the beat frequency induced by ADP was also observed in the spontaneous beating at 3.0 μM ATP by an application of 1–20 μM ADP, but the beat frequencies in the presence of ADP did not significantly change with the ADP concentration (Fig. 6C). We should also note that even in the flagella that were beating at 0.3 Hz or higher under imposed bending at 3.0 μM ATP (Table I), continuous beating stopped on removal of the mechanical constraint. Thus, there was no direct correlation between the beat frequency and the stability of beating. We may conclude that the beat frequency does not by itself determine the stability of oscillation, and that the presence of ADP seems to be important for the regulation of the rate as well as the stable switching of the microtubule sliding during flagellar beating.

Roles of the proximal region of flagella in oscillation

To determine whether the proximal region of flagella is important for the flagellar responses to mechanical perturbations, we studied the effect of bending the more distal region. To do this we attached both the head and a region of the flagellum just behind the head to the microneedle. The length of the attached part of the flagella was varied over the range 2–32 μm. As the average length of flagella was 40.7±2.6 μm (n=55), the length of the free region of the flagellum (L in Fig. 7A) that was experimentally bent was about 3–41 μm.

At 2.0 μM ATP, bending the whole flagella induced a pair of bends ("bend formation" in Fig. 7B). In most cases this was followed by "propagation" (35%), a "full cycle" (20%) or "beating" (35%), but preventing the movement of the proximal region decreased the frequency of development of these flagellar responses (Fig. 7B). The shorter the length of the free portion of the flagellum, the lower the frequency of occurrence of the more developed types of response. For the induction of "beating", the proximal half or so of the flagellum was required.

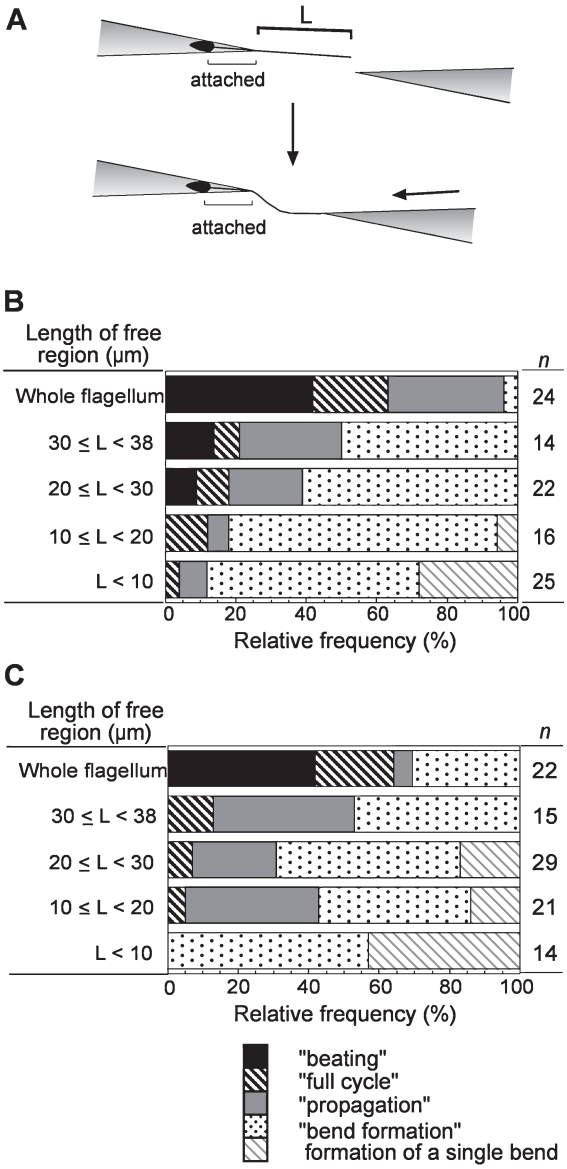


Fig. 7. Effects of the length of the flagellum that can be deformed on the induced flagellar response. (A) The proximal part of the flagellum was attached to the microneedle (left) along with the sperm head (top). The free region of the flagellum (L) was bent by moving the right microneedle (bottom). (B) and (C) summarizes the rate of occurrence of the flagellar responses at 2.0 μM ATP (B) and 1.5 μM ATP with 1.5 μM ADP (C). The length of the free region, L, was measured as the distance measured along the flagellum between the tips of the left and the right microneedles. The whole lengths of the flagella used were about 41 μm.

We also tested the effect of preventing the movement of the proximal region in the presence of ADP. Fig. 7C shows the occurrence of the flagellar responses in the presence of 1.5 μM ATP and 1.5 μM ADP. Without prevention, some bends developed "beating" (Figs. 5 and 7C) and the percentage of the "beating" (40%) was similar to that observed in the whole flagella at 2.0 μM ATP (Fig. 7B). Preventing the

movement of the proximal region for only 2–3 μm, however, reduced the percentage of "beating" to zero (Fig. 7C).

Discussion

In this study we have shown that the demembranated flagella of sea urchin sperm are capable of beating by responding to imposed bending or a mechanical pulse at ATP concentrations around and below the threshold for spontaneous beating. We further identified several factors determining the induction of oscillation: 1) The critical (threshold) concentration of ATP is 2.0 μM; 2) the presence of ADP reduces the threshold to 1.5 μM ATP; 3) localized microtubule sliding in the flagellum to form a pair of bends is necessary; 4) the axial difference along the flagellum is important.

Threshold ATP concentration for oscillation

The threshold ATP concentration for the mechanically induced beating was 2.0 μM ATP (or 1.65 μM MgATP), which was similar to that for spontaneous beating. Four types of flagellar response were induced in motionless flagella in an ATP concentration-dependent manner and the beat frequency of the induced beating was also ATP-dependent. These observations suggest that the mechanically induced oscillation is caused by the activity of dynein under the same regulatory mechanism as the one that underlies normal oscillatory beating. The presence of a threshold ATP concentration for beating indicates that a certain proportion of dynein arms engaged in force production is required for the induction of microtubule sliding in the axoneme. The threshold ATP concentration for beating may be the minimum concentration of ATP necessary for the coordinated activity of dynein arms, which may involve a regulatory mechanism as well as force production of dyneins.

The ATP concentration required for the reactivation of beating is slightly higher in a reactivating solution containing KCl (8–10 μM) (Shingyoji *et al*., 1977) instead of K-acetate (2 μM in this study). It has been reported that the demembranated flagella show a higher beat frequency in a reactivating solution containing K-acetate instead of KCl (Gibbons *et al.*, 1982). In general, acetate anions have a stronger stabilizing effect on the structure and function of proteins than chloride anions (Cacace *et al*., 1997); the different properties of these anions could affect the activation of dynein. The stability of dynein molecules would be important for the regulation of their motile function. Stabilizing ions may elevate the level of coordination between the dynein molecules, resulting in a decrease of the threshold ATP concentration for beating. It has been demonstrated that the presence of free Mg^{2+} and ATP^{4-} at the mM level influences the dynein activity and decreases the beat frequency (Okuno and Brokaw, 1979). The concentrations of free Mg^{2+} (1.0 mM) and ATP^{4-} (0.3–1.5 μM) in this study should not change over a broad range of ATP concentrations (1.5–10 μM). Therefore, we conclude that the stoppage of beating at a very low ATP concentration is caused by the lack of ATP: ATP above the threshold concentration is necessary for the coordinated activity of dynein arms to produce flagellar oscillation.

The importance of mechanical signals in the regulation of flagellar movement has been pointed out by previous studies, which suggested that the mechanical manipulation enhances the flagellar motility (Okuno and Hiramoto, 1976; Shingyoji *et al.*, 1991; Shingyoji *et al.*, 1995; Hayashibe *et al*., 1997; Morita and Shingyoji, 2004). In these studies, changes of the beat frequency and bend angle of beating flagella were induced by application of a continuous mechanical signal (imposed bending), while beating was achieved by a transient signal (mechanical pulse). Both continuous and transient signals were found to be useful to induce oscillatory beating in this study. The result indicates an important role of the mechanical signal in triggering the coordinated activity of dynein to produce microtubule sliding. Besides the increase of the dynein activity, imposed bending was effective to induce the switching of microtubule sliding necessary for the alternation of bending directions (Morita and Shingyoji, 2004). This seems to be related to the self-regulatory feedback system by which the motile activity of dynein is regulated to produce oscillatory movement. The present finding that the oscillatory beating can be induced in motionless flagella by imposed mechanical signals strongly supports the idea that mechanical signals are involved in the self-regulatory feedback system underlying flagellar movement.

Importance of ADP in flagellar oscillation

ADP is known to play an important role in the regulation of the motile activity of dynein and flagellar movement (Lindemann and Rikmenspoel, 1972a, 1972b). ADP has been shown to be a factor enabling the non-motile *Chlamydomonas* flagella missing a part of the central pair or radial spokes to beat (Omoto *et al*., 1996). ADP also increases the velocity of microtubule sliding by isolated inner arm dynein (Yagi, 2000; Shiroguchi and Toyoshima, 2001; Kikushima *et al*., 2004). The motile activity of cytoplasmic dynein has been shown to be regulated by the condition of nucleotide binding (Kon *et al*., 2004). If this is also the case for axonemal dynein at low ATP, a very small amount of ADP can be important for the regulation of motile activity.

The present finding has shown that ADP is an activator of the motile function of dynein and strongly supports the above-mentioned studies: Responding to mechanical signals, demembranated flagella started oscillatory beating even at 1.5 μM ATP (or 1.2 μM MgATP) which was below the threshold, only when ADP was present. ADP possibly

changes the structure and function of dynein to enable it to generate microtubule sliding in response to the mechanical signal. The beat frequency of the induced oscillation did not change over a wide range of ADP concentrations (1.5–10.0 μM ADP or 0.7–4.6 μM MgADP). The effect of ADP cannot be explained simply by an increase of the ATP concentration.

The induction of oscillation seems to be determined not directly by ATP but by a combination of ATP and ADP. The present observations appear to be closely related to the behavior of a monomeric inner arm dynein of *Tetrahymena*, whose ATPase activity and the microtubule translocation activity follow the Michaelis-Menten type kinetics only in the presence of a low concentration of ADP (Shiroguchi and Toyoshima, 2001). The mechanism by which ADP modulates the activity of dynein has not been elucidated, but a tempting possibility would be as follows: the mechanical signals accelerate the motile activity of dynein by increasing the binding of ADP to non-catalytic nucleotide binding sites of dynein. How ADP interacts with (or binds to) dynein under physiological ATP conditions is one of the next questions of interest.

Paired bend formation as a key step

Previous studies have shown that localized, cyclical formation of a pair of bends can be brought about by ATP iontophoresis in any region of the flagellum (Shingyoji *et al.*, 1977; Takahashi *et al*., 1982). This demonstration shows that the ability to generate oscillation is distributed along the flagellum and not restricted to a specific region, e.g., at the flagellar base, and that local difference of the microtubule sliding along the flagellum is the basis for bend formation. In the present study, formation of a pair of bends by mechanical signals was required for further development including bidirectional bend formation (a "full cycle") and oscillation ("beating"). The bending and the mechanical pulse used in this study induced similar responses. Dynein may have received different mechanical signals in these procedures, but the result indicates that the initial transient probably functioned as the signal to induce microtubule sliding to form a pair of bends.

Detailed analysis of the starting transients induced by an increase of pH (from 5.3 to 8.2) in live sperm or by an application of 0.2 mM ATP in demembranated sperm flagella have shown that the straight flagella begin to move by synchronous sliding, forming a basal bend, while flagella that contain a basal bend begin to move by metachronous sliding within that bend (Goldstein, 1979). When a sperm axoneme is subjected to bath application of ATP the synchronous sliding all along the flagellum would be followed by metachronous sliding at the basal bend, which would trigger continuous beating. Similar response, in which mechanically induced synchronous sliding triggers beating, has been reported in paralysed *Chlamydomonas* flagella missing the central pairs or radial spokes. Beating can be induced in these flagella when forced bending is applied so as to form a single bend at the base (Hayashibe *et al*., 1997). In the present study, mechanical deformation at a very low ATP concentration produced a basal bend probably by synchronous sliding (Fig. 2A) which, however, was not followed by metachronous sliding. In contrast, when a pair of bends was induced by bending, synchronous sliding occurred at the interbend region and metachronous sliding occurred at the bends. Furthermore, the present results indicate that the metachronous sliding at the distal bend of a pair of bends is important for the development of the bends, since in the "full cycle" and the "beating" the growth and propagation of the bend occurred first at the distal bend and then at the proximal bend. This result indicates a possible role of the metachronous sliding at the distal bend in regulating the dynein activity at the more proximal bend.

Roles of axial difference of flagella

The ability of generating oscillation has also been demonstrated to be distributed along the flagella by laser-beam amputation experiments (Goldstein *et al*., 1970) and by locally inhibiting microtubule sliding with an inhibitor (Fujimura and Okuno, 2006). These observations have suggested that beating can occur if the microtubules are tied together at one fixed end of the flagellum. This "basal anchoring model" is also supported by computer simulation (Brokaw, 1986) and by theoretical models (Lindemann, 1994; Lindemann and Kanous, 1995). In the present study, a microneedle was attached to the proximal region of the flagellum which prevented bending movement, but probably did not completely inhibit microtubule sliding, in this region. We found that mechanical induction of a pair of bends in the middle region of the flagellum produces "beating", indicating that the fixed basal end is not essential for flagellar oscillation. It seems that the formation of a pair of bends, which results from spatial differences in microtubule sliding along the flagellum is essential. However, oscillatory movement was not induced in the distal half of the flagellum even when a pair of bends is induced by mechanical deformation. Thus, induction of microtubule sliding to form a pair of bends is not enough for the initiation of oscillation.

The regional difference along the flagellum is consistent with the incomplete wave propagation observed in the distal region at a low ATP concentration, such as 3 μM ATP. The observed difference between the proximal and the distal regions may be caused by a difference in the types of dynein arms along the flagellum (Piperno and Ramanis, 1991) although there is no clear evidence supporting this idea in sea urchin sperm. It is also possible that the spatial difference is caused by differences in structures other than dynein, e.g. the central pair/radial spoke complex, which are important for the regulation of dynein activity.

The mechanical deformation (or imposed bending) used

in this study is a useful method for inducing stable, reproducible responses. To remove the mechanical signal, however, the microneedle attached to the distal end of the flagellum should be detached, which inevitably gives a new mechanical signal to the flagellum. The presence of a small amount of ADP (1.5–10.0 μM ADP or 0.7–4.6 μM MgADP), however, enables the flagellum to beat even during and after the removal of the microneedle. The mechanism of activation of dynein by ADP is not understood. If ADP behaves as a positive regulator of dynein activity, a possible interpretation is that the mechanical signal activates the motile function of dynein through the interaction of ADP with the dynein arms.

Whether mechanisms similar to what we have found under low ATP conditions operate in live sperm or in reactivated sperm flagella under high ATP conditions remains to be elucidated. In all cases, axial differences along the flagellum should be considered as a factor regulating the activity of dynein during flagellar oscillation. This difference would generate a spatial difference in microtubule sliding along the flagellum to cause bend formation. Formation of bends in distal regions will trigger microtubule sliding in more proximal regions, leading to further formation of bends (Morita and Shingyoji, 2004) and thus initiating flagellar oscillation.

Acknowledgements. We would like to express our gratitude to Professor Keiichi Takahashi for discussion and improving of the manuscript. This work was supported by Grant-in-Aid for Scientific Research on Priority Area from the Ministry of Education, Culture, Sports, Science and Technology, the Japanese Government (C.S.).

References

Brokaw, C.J. 1975. Effects of viscosity and ATP concentration on the movement of reactivated sea-urchin sperm flagella. *J. Exp. Biol*., **62**: 701–719.

Brokaw, C.J. 1986. Computer simulation of bend propagation by axoplasmic microtubules. *Cell Motil. Cytoskel.*, **6**: 347–353.

Brokaw, C.J. 1991. Microtubule sliding in swimming sperm flagella: direct and indirect measurements on sea urchin and tunicate spermatozoa. *J. Cell Biol.*, **114**: 1201–1215.

Cacace, M.G., Landau, E.M., and Ramsden, J.J. 1997. The Hofmeister series: salt and solvent effects on interfacial phenomena. *Q. Rev. Biophys*., **30**: 241–277.

Fujimura, M. and Okuno, M. 2006. Requirement of the fixed end for spontaneous beating in flagella. *J. Exp. Biol*., **209**: 1336–1343.

Gibbons, B.H. and Gibbons, I.R. 1972. Flagellar movement and adenosine triphosphatase activity in sea urchin sperm extracted with Triton X-100. *J. Cell Biol.*, **54**: 75–97.

Gibbons, B.H. and Gibbons, I.R. 1974. Properties of flagellar "rigor waves" formed by abrupt removal of adenosine triphosphate from actively swimming sea urchin sperm. *J. Cell Biol.*, **63**: 970–985.

Gibbons, I.R., Evans, J.A., and Gibbons, B.H. 1982. Acetate anions stabilize the latency of dynein 1 ATPase and increase the velocity of tubule sliding in reactivated sperm flagella. *Cell Motil. Suppl.*, **1**: 181–184.

Gibbons, I.R., Shingyoji, C., Murakami, A., and Takahashi, K. 1987. Spontaneous recovery after experimental manipulation of the plane of beat in sperm flagella. *Nature*, **325**: 351–352.

Goldstein, S.F., Holwill, M.E., and Silvester, N.R. 1970. The effects of laser microbeam irradiation on the flagellum of *Crithidia* (*Strigomonas*) *oncopelti*. *J. Exp. Biol*., **53**: 401–409.

Goldstein, S.F. 1979. Starting transients in sea urchin sperm flagella. *J. Cell Biol*., **80**: 61–68.

Hayashibe, K., Shingyoji, C., and Kamiya, R. 1997. Induction of temporary beating in paralyzed flagella of *Chlamydomonas* mutants by application of external force. *Cell Motil. Cytoskel.*, **37**: 232–239.

Kikushima, K., Yagi, T., and Kamiya, R. 2004. Slow ADP-dependent acceleration of microtubule translocation produced by an axonemal dynein. *FEBS Lett*., **563**: 119–122.

Kinoshita, S., Miki-Noumura, T., and Omoto, C.K. 1995. Regulatory role of nucleotides in axonemal function. *Cell Motil. Cytoskel.*, **32**: 46–54.

Kon, T., Nishiura, M., Ohkura, R., Toyoshima, Y.Y., and Sutoh, K. 2004. Distinct functions of nucleotide-binding/hydrolysis sites in the four AAA modules of cytoplasmic dynein. *Biochemistry*, **43**: 11266–11274.

Lindemann, C.B. and Rikmenspoel, R. 1972a. Sperm flagella: Autonomous oscillations of the contractile system. *Science*, **175**: 337–338.

Lindemann, C.B. and Rikmenspoel, R. 1972b. Sperm flagellar motion maintained by ADP. *Exptl. Cell Res*., **73**: 255–259.

Lindemann, C.B. 1994. A "Geometric Clutch" hypothesis to explain oscillations of the axoneme of cilia and flagella. *J. Theor. Biol.*, **168**: 175–189.

Lindemann, C.B. and Kanous K.S. 1995. "Geometric clutch" hypothesis of axonemal function: key issues and testable predictions. *Cell Motil. Cytoskel.*, **31**: 1–8.

Morita, Y. and Shingyoji, C. 2004. Effects of imposed bending on microtubule sliding in sperm flagella. *Curr. Biol.*, **14**: 2113–2118.

Okuno, M. and Hiramoto, Y. 1976. Mechanical stimulation of starfish sperm flagella. *J. Exp. Biol*., **65**: 401–413.

Okuno, M. and Brokaw, C.J. 1979. Inhibition of movement of Triton-demembranated sea-urchin sperm flagella by Mg^{2+}, ATP^{4-}, ADP and Pi. *J. Cell Sci.*, **38**: 105–123.

Omoto, C.K., Yagi, T., Kurimoto, E., and Kamiya, R. 1996. Ability of paralyzed flagella mutants of *Chlamydomonas* to move. *Cell Motil. Cytoskel.*, **33**: 88–94.

Piperno, G. and Ramanis, Z. 1991. The proximal portion of *Chlamydomonas* flagella contains a distinct set of inner dynein arms. *J. Cell Biol.*, **112**: 701–709.

Sale, W.S. and Satir, P. 1976. Splayed *Tetrahymena* cilia. A system for analyzing sliding and axonemal spoke arrangements. *J. Cell Biol.*, **71**: 589–605.

Shingyoji, C., Murakami, A., and Takahashi, K. 1977. Local reactivation of Triton-extracted flagella by iontophoretic application of ATP. *Nature*, **265**: 269–270.

Shingyoji, C., Gibbons, I.R., Murakami, A., and Takahashi, K. 1991. Effect of imposed head vibration on the stability and waveform of flagellar beating in sea urchin spermatozoa. *J. Exp. Biol*., **156**: 63–80.

Shingyoji, C., Yoshimura, K., Eshel, D., Takahashi, K., and Gibbons, I.R. 1995. Effect of beat frequency on the velocity of microtubule sliding in reactivated sea urchin sperm flagella under imposed head vibration. *J. Exp. Biol*., **198**: 645–653.

Shiroguchi, K. and Toyoshima, Y.Y. 2001. Regulation of monomeric dynein activity by ATP and ADP concentrations. *Cell Motil. Cytoskel*., **49**: 189–199.

Summers, K.E. and Gibbons, I.R. 1971. Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. *Proc. Natl. Acad. Sci. USA*, **68**: 3092–3096.

Takahashi, K., Shingyoji, C., and Kamimura, S. 1982. Microtubule sliding in reactivated flagella. In *Prokaryotic and Eukaryotic Flagella* (W.B. Amos, and J.G. Duckett, eds.). Cambridge University Press, Cambridge, pp.159–177.

Yagi, T. 2000. ADP-dependent microtubule translocation by flagellar inner-arm dyneins. *Cell Struct. Funct*., **25**: 263–267.

Yoshimura, M. and Shingyoji, C. 1999. Effects of the central pair apparatus on microtubule sliding velocity in sea urchin sperm flagella. *Cell Struct. Funct*., **24**: 43–54.

(Received for publication, November 30, 2006
and accepted, January 17, 2007)