The Journal of Experimental Biology 209, 1336-1343
Published by The Company of Biologists 2006
doi:10.1242/jeb.02131

Requirement of the fixed end for spontaneous beating in flagella

Miki Fujimura and Makoto Okuno*

Department of Life Sciences, Graduate School of Arts and Sciences, University of Tokyo, Meguro-ku, Tokyo 153-8902, Japan

*Author for correspondence (e-mail: cokuno@mail.ecc.u-tokyo.ac.jp)

Accepted 26 January 2006

Summary

It is well known that any part of a flagellum has the ability to bend. However, it is not clearly understood how flagella generate successive bending waves spontaneously. Some micromanipulation experiments have suggested that the base of the flagellum is required. By contrast, spontaneous bending waves could be generated in computer simulation work if the microtubules were tied together at one end. We hypothesized that the basal structure of flagella can only act as a tied end when the outer doublet microtubules are tightly bound together so as not to slide. We developed a new technique for introducing local inhibition at any position on the demembranated and reactivated flagellum. The flagellum maintained spontaneous beating when the local inhibition was introduced at any position on it. In addition, spontaneous beating occurred without the basal body when an artificial fixed region was introduced to the flagellum. We conclude that the axoneme, a bundle of microtubules, requires the fixed end for spontaneous beating.

Key words: flagellar movement, local inhibition, PRODAN, sea urchin.

Introduction

The flagellum is a micro-machine that produces spontaneous beating. The bending wave is the product of active sliding among the outer doublet microtubules, generated by dynein ATPase. In metazoa, dynein is composed of two heavy chains, three to four intermediate chains and several light chains. However, little is known about the mechanism involved in generation and propagation of the bending wave, and there is no appropriate explanation for the relationship between active sliding of the outer doublet microtubules and spontaneous bending wave generation.

Several experiments have been performed in order to gain deeper understanding of these mechanisms. When an actively beating sperm flagellum was cut by a laser beam, pre-existing waves in the distal part continued to propagate. The distal part, however, lost the ability to produce a new wave, whereas the proximal part of flagellum connected to the head continued beating (Goldstein, 1969). Similar results were obtained in short flagella dissected mechanically by homogenization (Gibbons, 1974) and in starfish sperm flagella arrested by a glass needle (Okuno and Hiramoto, 1976). These results suggest that the basal part of flagella is necessary to produce spontaneous beating.

Every part of demembranated flagella could bend when ATP was applied locally to flagella in the rigor state (see below) by iontophoresis (Shingyoji et al., 1977). Although successive pulse application of ATP did cause successive progression of the waveform all along the flagella (Shingyoji and Takahashi, 1995), it was not concluded that spontaneous beating can occur at any position on the flagella because the preparation used was very different from intact sperm flagella. In contrast to live sperm flagella, demembranated flagella stayed in the 'rigor state', in which dynein arms build tight cross-bridges among microtubules because of the lack of ATP, except in the area exposed to ATP in that experiment. How is the bending wave spontaneously generated, propagated through the flagellum, and maintained stably?

In his series of computer simulation works, Brokaw demonstrated that simulated flagella continued to develop, propagate and maintain the bending waves under various conditions if the microtubules were tied together at one end (Brokaw, 1986). If this is also true for real flagella, spontaneous beating could occur at any site on the flagellar axoneme when one end is fixed. This 'basal anchoring' model is supported by Lindemann in his 'Geometric Clutch' hypothesis (Lindemann, 1994; Lindemann and Kanous, 1995). Lindemann considered that basal anchoring of the flagellum provides the tension on the doublet, and this tension is necessary to provide the transverse force required for switching the activity of dynein. Woolley and Bozkurt reported that dissected sperm flagella produced beating (Woolley and Bozkurt, 1995), although it lasted for only a short period. They also found that compression of the proximal end of the dissected flagellum made it possible to produce bends. Thus,

they confirmed the basal region of flagellum as the fixed end that generates resistance against sliding of microtubules. Their experiments, however, failed to maintain the stable beating for a long time. In addition, it was not evident that the compression of the axoneme by the microneedle could tie 9+2 microtubules together. The bending wave shown in their paper was of small amplitude and low beat frequency, as we previously observed with amputated starfish sperm flagella (Okuno and Hiramoto, 1976) compared with reactivated intact flagella. So far, it has not been established whether any part of the flagellum has the potential to generate and maintain a normal bending wave of large amplitude spontaneously and stably. The necessity for the basal region of the flagellum to act as 'pacemaker-like machinery' for normal beating with high beat frequency and large amplitude cannot be eliminated.

To solve this problem, we wished to inhibit flagellar movement locally. We aimed to develop a new technique that would introduce a fixed narrow region where sliding between microtubules could not occur. If successful, we could, for example, examine whether movement of the distal region was arrested or maintained when movement of the basal region was inhibited. If movement was arrested, then spontaneous beating must require some kind of 'signal' transmitted from the 'pacemaker-like machinery' at the base of the flagellum. By contrast, if movement was maintained, then there would be no need for the 'pacemaker-like machinery'. Until now, however, it has been very difficult to test this idea since application of inhibitors to a limited area of flagella is technically difficult because of diffusion of the inhibitor itself.

In the present study, we identified a fluorescent reagent, PRODAN (6-propionyl-2-dimethylamino-naphtalene), which inhibits flagellar movement only after excitation by UV irradiation. PRODAN treatment and spot irradiation after excitation with UV light successfully caused a local inhibition of flagellar movement.

Materials and methods

Preparation of demembranated spermatozoa

Sperm of sea urchin *Anthocidaris crassispina* L. were demembranated and reactivated according to the method of Okuno and Brokaw (Okuno and Brokaw, 1979) with a little modification. Briefly, sea urchin spermatozoa were suspended in 4 volumes of Ca^{2+}-free artificial seawater containing 465 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 25 mmol l^{-1} $MgSO_4$, 0.2 mmol l^{-1} EDTA and 2 mmol l^{-1} Tris-HCl, pH 8.2. The suspended spermatozoa were demembranated with 40 volumes of demembranating solution and reactivated with the reactivating solution. The demembranating solution contained 0.04% (w/v) Triton X-100, 0.2 mol l^{-1} potassium acetate, 2 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} EGTA, 2 mmol l^{-1} Tris-HCl, pH 8.2), and 2 mmol l^{-1} dithiothreitol (DTT). The reactivating solution contained 0.2 mol l^{-1} potassium acetate, 2 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} EGTA, 20 mmol l^{-1} Tris-HCl, pH 8.2, 2% (w/v) polyethylene glycol, 1 mmol l^{-1} DTT and various concentrations of ATP.

PRODAN (6-propionyl-2-dimethylamino-naphtalene; Molecular Probes, Eugene, OR, USA) was applied to the demembranated spermatozoa, by transferring them into the reactivation solution without ATP, then incubating with 10 μmol l^{-1} dye for 2 min on ice. The spermatozoa were then reactivated by adding an appropriate volume of solution containing ATP.

Observation and analysis of flagellar movement

Reactivated sperm suspension was poured into the observation chamber, which consisted of two strips of vinyl tape on the slide glass covered with a cover glass. The depth of the chamber was changed according to the experiment. Thin tape was used for observation only or for perfusion experiments. Thick tape was used for the micromanipulation experiment described later, in which a glass microneedle was inserted through the side openings of the chamber.

Reactivated spermatozoa were observed and recorded using phase-contrast or dark-field, and fluorescence microscopy (BX-51, Olympus, Tokyo, Japan). The microscope was equipped with a video camera (63V1N, Mintron, or CR-20, Video-device, Tokyo, Japan) and a video tape recorder (BR-S800, Victor, Yokohama, Japan). The objective lens was UplanFl (40×, NA 0.75, Olympus, Tokyo, Japan). Video-recorded images were captured by Storm Video Version 1.00 (Canopus, Kobe, Japan). Shear angle was analyzed from video recordings by 'Bohboh', a flagellar movement auto-analyzing software kindly provided by Dr Baba. Shear angle was defined as the angle between the tangent at the base of the flagellum and that at any point along the flagellum, and was assumed to be proportional to the amount of microtubule sliding.

Local irradiation of UV

Local UV irradiation was applied to the demembranated flagellum using fluorescence microscopy (Olympus BX-51). The microscope was equipped with a 100 W ultra high-pressure mercury lamp (USH102D, Ushio, Tokyo, Japan) and U-MWU2 (Olympus, Tokyo, Japan) filter-box for irradiation of UV light (330–385 nm) to the specimen and for observation. We put a pinhole at the position of the iris on the optical path for the mercury lamp and could thereby irradiate UV light to a very restricted area (minimum 2.5 μm in diameter). The strength of UV was adjusted by ND filters (U-25ND6, U-25ND25, U-25ND50, Olympus, Tokyo, Japan).

The irradiated area was marked on a flat TV-monitor before the inhibition experiment was carried out, and the PRODAN-treated flagellum was moved to the marked area for UV irradiation of the intended portion.

Trypsin treatment of flagella

Spermatozoa were demembranated and reactivated in the presence of PRODAN. The reactivated sperm suspension was poured into the observation chamber for UV irradiation, after ensuring that sperm were beating in the focal plane with the head attached to the glass surface. Then, the chamber was perfused with reactivating solution containing 0.2 μg ml^{-1}

trypsin. After an appropriate time, when microtubules had completed sliding out from the axoneme, the trypsin was washed out by reactivating solution without trypsin, and photographs were taken in order to assess the microtubule disintegration patterns.

Dissection of flagella

Glass microneedles were made using a pipette puller (PG-1, Narishige, Tokyo, Japan) from thin glass rods about 1 mm in diameter, and were held by a micromanipulator (MO-102, Narishige, Tokyo, Japan). A head-attached and PRODAN-treated spermatozoon beating in the focal plane was displaced to almost the center of the microscope field. After spot UV treatment, the middle of the UV-irradiated area (the motility-inhibited region) of the flagellum was cut by pressing a glass microneedle onto the coverslip.

Temperature

All experiments were carried out at room temperature 23±2°C.

Reagents

PRODAN was purchased from Molecular Probes; all other chemicals were from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Results

Development of a new technique that inhibits flagellar movement locally

In the present study, we report a new technique for local inhibition of flagellar movement. We thought that if we could find a fluorescent reagent that could bind to the axoneme and inhibit flagellar movement only after excitation of the dye by UV light, spot irradiation by the excitation light should cause local inhibition of the flagellum. A fluorescent reagent, PRODAN (6-propionyl-2-dimethylamino-naphtalene), provided the feature we expected. It inhibited flagellar movement only when the dye was excited by UV light, as shown below.

PRODAN was introduced by Weber and Farris in 1979 (Weber and Farris, 1979) and has generally been used as a membrane surface marker. It has also been used as a non-covalently interacting probe for proteins (Hiratsuka, 1999). When the solvent is water, the excitation wavelength of the dye is 361 nm and the emission wavelength 531 nm. For UV irradiation, we employed a fluorescence microscope. A pinhole was put at the position of the iris on the optical path of the high pressure-mercury lamp to control the irradiation area, so that we could change the irradiation area by exchanging the pinhole with one of a different diameter.

Spermatozoa of the sea urchin *Anthocidaris crassispina* were demembranated with Triton X-100 and reactivated. The beat frequency and other features of the reactivated flagellar movement in Triton-extracted sperm were not affected by incubation with PRODAN alone (at concentrations of 10 μmol l^{-1} or less), since the motility of the reactivated sperm was maintained for more than 20 min without any change in wave parameters (data not shown).

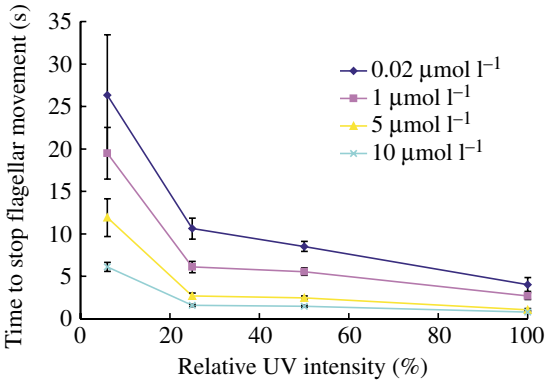

Fig. 1. Relationship between UV intensity and irradiation period for complete suppression of reactivated flagella. UV irradiation covered the entire flagellum and flagellar movement stopped completely within this period. The intensity of UV irradiation was defined as a relative value. Irradiation without the ND filter was defined as 100% (see Materials and methods). The concentration of PRODAN was 0.02 μmol l^{-1} (diamonds), 1 μmol l^{-1} (squares), 5 μmol l^{-1} (triangles) and 10 μmol l^{-1} (crosses).

We first irradiated the entire flagellum without the pinhole in the optic path. When reactivated flagella were incubated with UV-irradiated PRODAN, referred to as PRODAN-UV treatment, the bending movement of the flagella was rapidly suppressed. The suppression was dependent both on the intensity of the UV irradiation and the concentration of PRODAN (Fig. 1). Flagella failed to recover motility even when UV irradiation was stopped, suggesting that the inhibition was irreversible. We could not determine the absolute intensity of the UV light in this study, so we defined the experiments without the ND filter as '100%' in relative terms. We used 10 μmol l^{-1} PRODAN and 100% UV in all local inhibition experiments, and flagellar movement was inhibited within 2 s of treatment.

Inhibition of microtubule sliding by PRODAN-UV

Flagella lost their motility after PRODAN-UV treatment. We assumed this loss of motility was due to inhibition of the sliding between microtubules. To test this, we locally activated PRODAN by UV irradiation through a pinhole, and then treated the flagellum with trypsin. Brief treatment with trypsin should cause non-irradiated flagella to disintegrate by disrupting cross-linking proteins such as nexin among microtubules. However, trypsin should fail to cause this disintegration if the proteins cross-linked by PRODAN were dynein rather than nexin, since dynein is resistant to trypsin.

Fig. 2 shows the results of the experiment where PRODAN-UV treatment was carried out on the basal (Fig. 2A) or distal (Fig. 2B) regions of the flagellum, followed by perfusion by trypsin-containing reactivating solution. Arrowheads mark PRODAN-UV treated regions and arrows the microtubule that disintegrated.

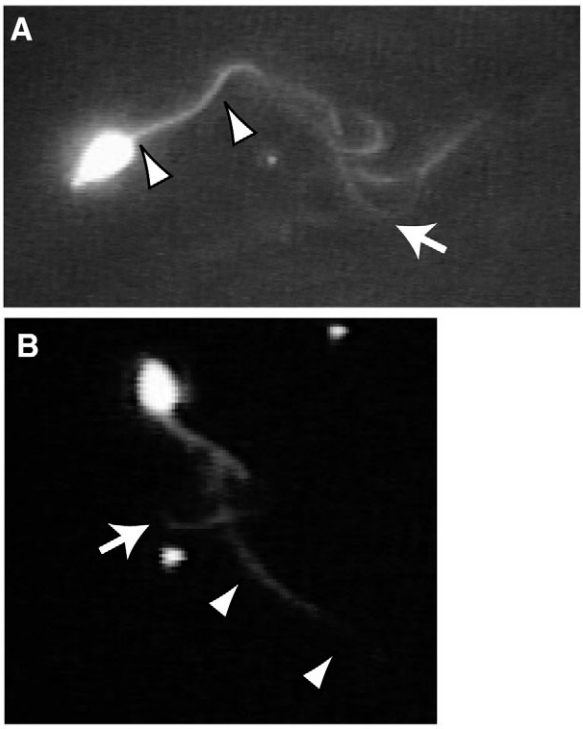

Fig. 2. Dark-field micrographs of flagella treated with PRODAN-UV 'locally' in the presence of ATP and trypsin. Limited disintegration of flagella was observed in the region without PRODAN-UV treatment. (A) Basal region of the flagellum treated with PRODAN-UV (the region between two arrowheads). (B) Distal region of a flagellum treated with PRODAN-UV (the region between two arrowheads). The arrow marks microtubules that came out loose.

In both the basal and distal regions, disintegration or spreading out was not observed at PRODAN-UV treated regions. By contrast, disintegration of microtubules was observed in the non-treated regions. When the whole flagellum was treated with PRODAN-UV, no disintegration or spreading out of microtubule(s) was observed at any part of the flagellum (data not shown), which often maintained the bent shape of 'rigor bends'. Therefore, we suggest that PRODAN-UV treatment inhibits sliding between microtubules by producing a tight cross-linker between microtubules.

Local inhibition of flagellar movement

We next investigated the effects of local UV irradiation on flagellar movement. To assess the inhibition, we measured the shear angle along the flagellum from video recordings. Shear curves represent the degree of bend in the flagellum at various distances along its length, and thus indicate the amount of microtubule sliding at each position (see Materials and methods). Fig. 3 shows photographs of wave-form and shear-curve analysis of a typical reactivated spermatozoon after the local UV irradiation, in the presence of 15 μmol l^{-1} ATP. The flagellum presented symmetrical bending waves before irradiation (Fig. 3A). When UV radiation was localized to the 5 μm region proximal to the flagellar base, no bend was observed in the irradiated area while the continuous beating was observed in the distal region (Fig. 3B). When the area of irradiation was extended to 12 μm in the same spermatozoon (Fig. 3C), the distal intact region still continued to generate the bending wave. The corresponding shear curves are shown in Fig. 3D–F, respectively.

In these studies, the beat frequency was observed to change from approximately 5 Hz to 8 Hz: 4.8 Hz (Fig. 3A,D), 5.0 Hz (Fig. 3B,E) and 8.0 Hz (Fig. 3C,F). The amplitude decreased simultaneously with the change in beat frequency of inhibited flagella. The result shown in Fig. 3 was a typical case of basal region inhibition, seen in over 50 experiments.

The relationship between beat frequency and length of the beating area of flagella is shown in Fig. 4. Flagella were gradually inhibited from their basal to distal regions and the beat frequency was measured as shown in Fig. 3. We defined 'movable length of flagellum' as the distance between the total length and inhibited length of the flagellum, in the presence of 10 μmol l^{-1} ATP. This experiment showed that the shorter the length of active flagellum, the higher the beat frequency. Therefore, it was likely that the beat frequency was determined not only by the ATP concentration but also the 'movable length' of flagella.

The sliding velocity of microtubules could be estimated by the product of the amount of sliding within one beat cycle times the beat frequency. This value is assumed to be proportional to the sliding velocity of microtubules. In Fig. 3, the values were approximately 10.3 rad s^{-1} for no inhibition (Fig. 3A,D), 8.7 rad s^{-1} for 5 μm inhibition (Fig. 3B,E), and 10.8 rad s^{-1} for 12 μm inhibition (Fig. 3C,F). Therefore, it was likely that the sliding velocity of microtubules in spontaneous beating remained constant at the fixed ATP concentration when the length of flagellum was changed.

When the distal part of a flagellum was subjected to UV irradiation, spontaneous beating continued in the intact area between the base and the irradiated distal part of the flagellum (data not shown).

The above experiments were carried out at low ATP concentration. When the ATP concentration was increased up to about 50 μmol l^{-1} or more, the flagellum features were dramatically changed. Typical results are shown in Fig. 5, in which ATP concentration was 0.2 mmol l^{-1}. When the distal part of flagella was subjected to UV irradiation, that area lost motility while the proximal region maintained motility (Fig. 5A). The result was almost equivalent to that observed at low concentrations of ATP. By contrast, when UV irradiation was performed at the proximal region, flagella lost motility in the intact distal region, as shown in Fig. 5B. Therefore, it was likely that the basal region of flagellum was necessary to generate and maintain the bending wave at high ATP concentrations.

Addition of cAMP, however, rescued the generation of bending waves in the distal area when the proximal area was inhibited by PRODAN-UV in the presence of a high concentration of ATP (Fig. 6). At low concentrations of ATP

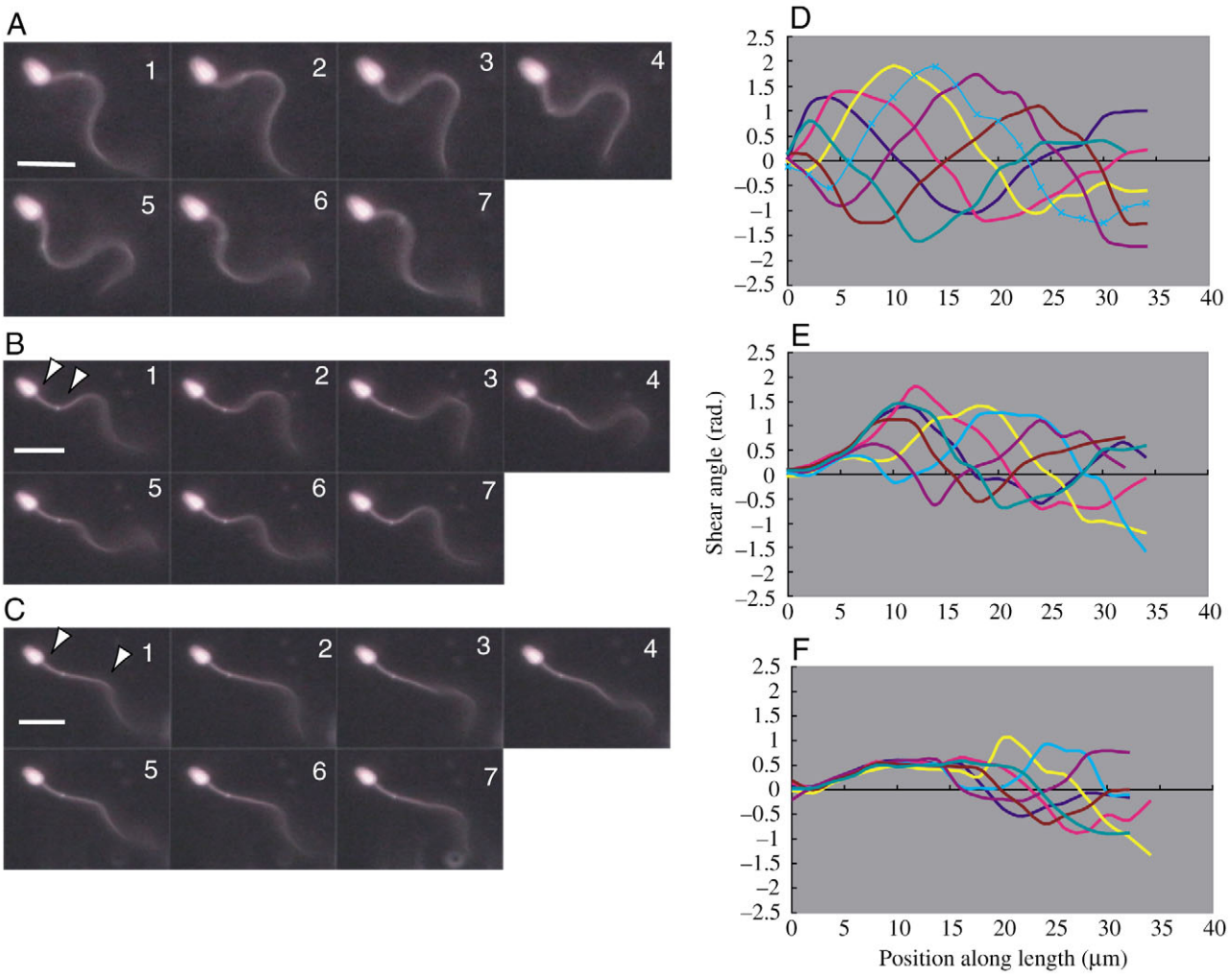

Fig. 3. Dark-field micrographs (A–C) and shear curves (D–F) of reactivated sea urchin sperm flagella. ATP concentration was 15 μmol l^{-1}. (A) Dark-field micrographs of symmetrical bending waves of the control sperm flagellum before UV irradiation. Beat frequency was 4.8 Hz. Bar, 10 μm. (B) Dark-field micrographs of 'proximal region inhibition step 1'. Approximately 5 μm of the proximal region was inhibited (between the two arrowheads). Beat frequency was 5.0 Hz. Bar, 10 μm. (C) Dark-field micrographs of 'proximal region inhibition step 2'. Approximately 12 μm of the proximal region was inhibited (between the two arrowheads). Beat frequency was 8.0 Hz. Bar, 10 μm. (D–F) Results obtained from the series of seven frames in A–C are superimposed in D–F, respectively. Every shear curve represents every 1/30 s. (D) Shear curves corresponding A. (E) Shear curves corresponding B. (F) Shear curves corresponding C.

(10 μmol l^{-1}), the intact portion showed spontaneous movement without addition of cAMP when the proximal region was inhibited, as shown in Fig. 3. At high concentrations such as 200 μmol l^{-1} ATP, however, about 95% of flagella showed no movement when the proximal regions were inhibited. Addition of cAMP overcame this inhibition in a concentration-dependant manner. At 200 μmol l^{-1} ATP, addition of 5 μmol l^{-1} cAMP improved the ratio of motile flagella from 5% to 60%. Addition of 50 μmol l^{-1} cAMP increased the ratio up to 70%. At 1 mmol l^{-1} ATP, flagella showed no sinusoidal bending wave when reactivating solution contained no cAMP. Addition of 50 μmol l^{-1} cAMP, however, caused about 20% of sinusoidal movement in the intact distal portion. Addition of 200 μmol l^{-1} cAMP improved the ratio of 'move' up to 40%.

Mechanical dissection of basal region reveals requirement of fixed end for spontaneous beating

The above experiments clearly demonstrate that any part of a flagellum has the potential to generate and maintain the spontaneous bending wave. However, some previous experiments (Goldstein, 1969; Okuno and Hiramoto, 1976) have suggested the possibility that a kind of 'control center' or 'pacemaker' exists at the base of flagellar axis that initiates the periodical beating, since the proximal part of flagellum is able to continue beating under various conditions such as cut-short or arrested flagella. In addition, it is also possible that a signal from the base of flagellum is transmitted to the distal area even when motility of the proximal part of flagellum is inhibited by PRODAN-UV. We therefore performed further experiments in which the base of the flagellum (including the head) was

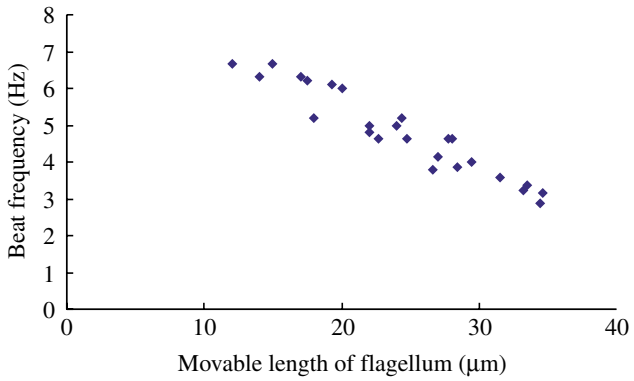

Fig. 4. Relationship between beat frequency and 'movable length' of the flagellum. The flagellum was inhibited in its proximal region in the presence of 10 μmol l^{-1} ATP.

dissected out from the principal part of the flagellum by a glass microneedle in order to examine whether the fixed end is necessary for generating and propagating the spontaneous bending wave. Fig. 7 shows a typical result in which the base of the flagellum was dissected out by pressing a glass microneedle onto the coverslip. When the basal region of demembranated flagellum was dissected out without PRODAN-UV treatment, we did not observe spontaneous generation of sinusoidal waves (data not shown). By contrast, when the basal region of flagella was subjected to PRODAN-UV treatment followed by dissection at the middle of its treated region, generation and propagation of spontaneous bending wave was maintained in the distal intact part. In Fig. 7B, the amplitude of the 'dissected' flagellar movement was a little smaller than of the intact one (see Fig. 3A). However, this phenomenon was commonly seen when the basal region was inhibited as shown in Fig. 3B,C, since the intact length of flagella had become shorter.

We therefore concluded that every part of a flagellum has the potential to generate and maintain the bending wave spontaneously when one end of the axoneme is fixed tightly, i.e. the bundle of microtubules was tightly cross-linked to provide an anchor point where the microtubules cannot slide.

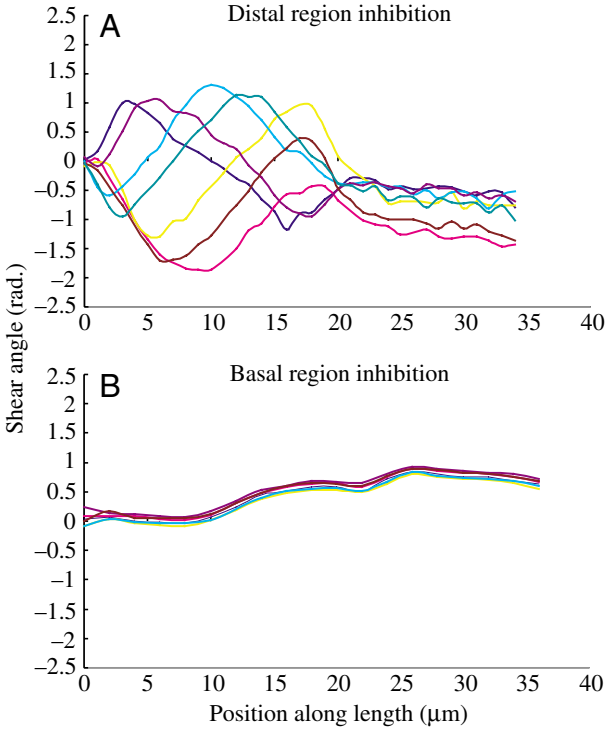

Fig. 5. UV irradiation of flagella reactivated with a high concentration of ATP (0.2 mmol l^{-1}). Shear curves from seven successive frames are superimposed. Every curve represents every 1/60 s. (A) Distal region (approximately 10 μm) inhibited. (B) Proximal region (approximately 10 μm) inhibited.

Discussion

In the present study, we succeeded in locally inhibiting flagellar movement and found that only a fixed end was necessary for spontaneous beating in flagella. No need for 'pacemaker-like' machinery was demonstrated.

Fluorescent reagent PRODAN as an inhibitor for flagellar movement

To achieve this local inhibition, we examined several fluorescent dyes that inhibited flagellar movement only on excitation of the dye. Some reagents other than PRODAN, such as 2,6-TNS (2-(p-toluidinyl)naphthalene-6-sulfonic acid) and 1,8-ANS (1-aminonaphthalene-8-sulfonic acid), had imilar features as inhibitors of flagellar movement. TNS and ANS are

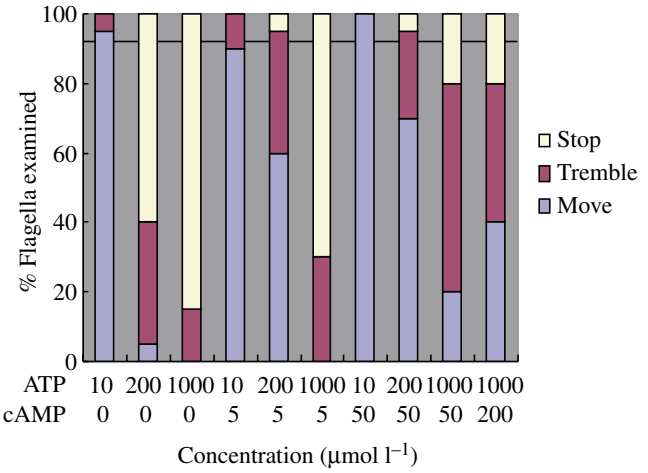

Fig. 6. Effect of cAMP on flagella inhibited by PRODAN-UV at the basal region (5–10 μm) in various concentrations of ATP and cAMP. MgATP concentrations were 10 μmol l^{-1}, 200 μmol l^{-1} and 1 mmol l^{-1}. cAMP concentrations were 0 μmol l^{-1}, 5 μmol l^{-1}, 50 μmol l^{-1} and 200 μmol l^{-1}. Key: move, intact distal portion shows sinusoidal, vigorous movement; tremble, intact portion does not show sinusoidal movement but does show slight vibration; stop, intact portion shows no movement. *N*=20 flagella examined for each condition.

both naphthalene sulfonates and bind to protein or membranes, like PRODAN. PRODAN, however, was the best inhibitor because of its strong inhibition and the fact that it was harmless to flagellar movement without UV irradiation.

Walker demonstrated a similar effect (Walker, 1961), the suppression of flagellar movement of a trypanosome by light irradiation and a fluorescent dye (acriflavine). However, his experiment was done with the intact trypanosome. Therefore, damage to the cell membrane might have caused inhibition of motility, since he reported local inhibition only on application of weak irradiation. High power irradiation caused complete suppression of flagellar motility. In the present study, by contrast, we succeeded in introducing the inhibition only at the restricted area of the axoneme subjected to UV irradiation.

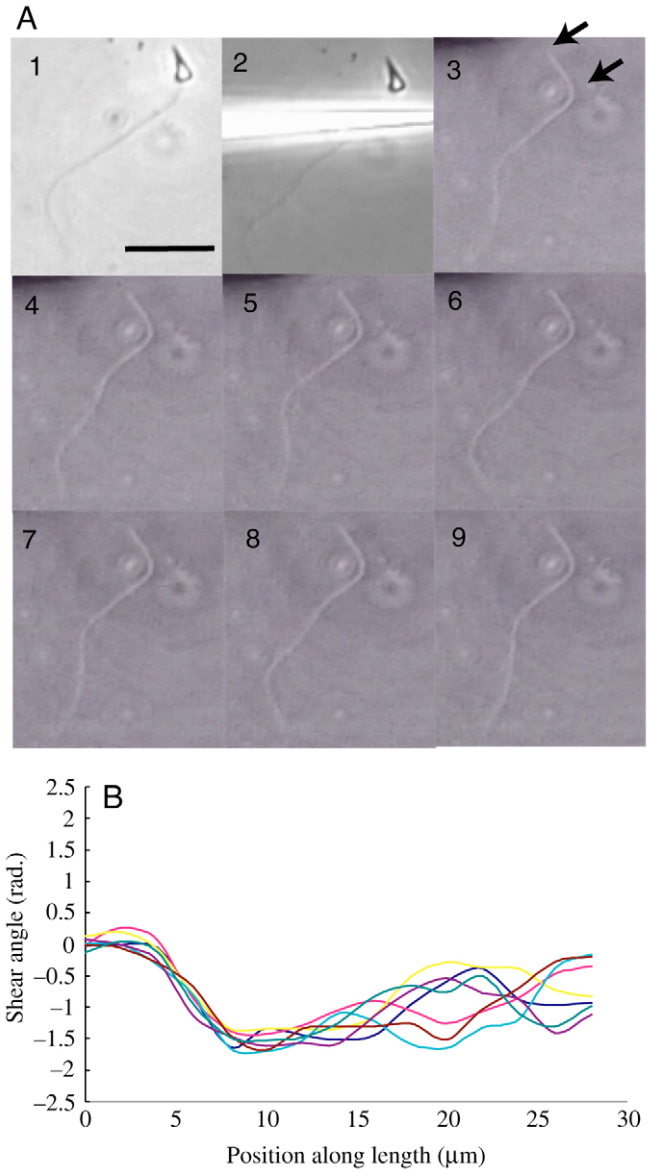

Fig. 7. Spontaneous beating of basal region dissected flagella. A head-attached, reactivated spermatozoon was treated with PRODAN-UV at the basal region of flagellum. Then, the PRODAN-UV treated basal region (between the arrows) was dissected out by a microglass needle. The dissected flagellum continued to show sinusoidal wave movement without a head and base of flagellum. This experiment was repeated more than 20 times. ATP concentration was 15 μmol l^{-1}. Bar, 10 μm. (A) Micrograph 1, the inhibited reactivated flagellum before its head was dissected out. Micrograph 2, the process of dissection. Micrograph 3–7, phase-contrast micrographs of the reactivated flagellum whose basal region (between two arrows) was PRODAN-UV treated and dissected out. Each micrograph represents 1/30 s. (B) Superimposed shear curves of the panels in A.

It was likely that PRODAN-UV treatment inhibited active sliding by constructing some tight cross-linking among microtubules. This conclusion was supported by observations that the inhibited flagella maintained a bent shape like ‘rigor bends’ and that the trypsinated flagella failed to disintegrate the microtubules either with extrusion or with spreading out in the presence of ATP (Fig. 2). What component(s) of flagella contributed to the formation of the tight connection among microtubules? The most plausible candidate at present could be dynein arms since the rigor state is introduced by cross-bridged dynein arms (Gibbons, 1975; Okuno, 1980). Other possible candidates might be nexin, radial spokes, and so on. If they were the cause, however, trypsinated flagella must have disintegrated since nexin and radial spokes are digested earlier than dynein so that dynein could work to promote active sliding of microtubules (Summers and Gibbons, 1971), and this should be explored using molecular level approaches.

Local inhibition of flagellar movement reveals the necessity of a tied end in the axoneme for spontaneous flagellar movement

In the present study, we demonstrate that any part of a flagellum has the potential to generate and maintain a bending wave when a tied end exists in the flagellar axoneme, although cAMP was also required at high concentrations of ATP. The basal body of the intact flagella could be substituted for the tied end introduced by PRODAN-UV in the present experiments. These results agree with Brokaw’s provision in his computer simulation work (Brokaw, 1986).

When the ATP concentration is high, the beat frequency of flagellar movement is high. It is likely that flagellar beating at high frequency requires ‘a coordination mechanism’ that functions with the assistance of cAMP. The requirement of cAMP for reactivation at high ATP concentrations has been demonstrated in salmonid fish sperm (Okuno and Morisawa, 1982) and sea urchin sperm (Ishiguro et al., 1982). The coordination mechanism should be distributed along the entire length of flagellum, not at the base of it, as shown in the present study. It could be assumed that cAMP works *via* A-kinase, resulting in phosphorylation of proteins such as dynein light chains in the flagellar axoneme (Inaba, 2003). In the present study, however, the concentration of cAMP required for spontaneous beating at high ATP concentration was much higher (Fig. 6). Therefore, the effect of cAMP might be different from the activation of A-kinase, and this requires further investigation.

It has been discussed whether the basal region of flagellum has a 'pacemaker-like' function or acts only as a fixed end to generate basal resistance that evokes bending (Brokaw, 1986; Lindemann, 1994). The pacemaker hypothesis is based on the results of experiments indicating that the flagellum shows no sinusoidal beating without its basal region. By contrast, the basal anchoring hypothesis was originally proposed from computer simulation, since experimental evidence was not available because of technical difficulties. In the present study, we introduced an 'artificial fixed end' into the flagellum and dissected out the middle of it. If the basal region of a flagellum has 'pacemaker-like' function, the dissected flagellum could not maintain a bending wave because it could not receive any kind of 'signal' transmitted from the 'pacemaker'. If the basal region of a flagellum bears only a fixed end function that generates basal resistance, the dissected flagellum should maintain its bending wave without its basal region.

The results of the present experiments show that the latter is the answer. A flagellum with an introduced artificial fixed end generated and maintained a spontaneous bending wave like an 'intact' demembranated flagellum. This result agrees well with the Brokaw's computer simulation (Brokaw, 1986) and Lindemann's 'Geometric Clutch' hypothesis (Lindemann, 1994; Lindemann and Kanous, 1995).

Factors for determining the beat frequency of flagella

It was thought that the beat frequency of flagellar movement is determined predominantly by the ATP concentration (Okuno and Brokaw, 1979). However, micromanipulation studies demonstrated that the beat frequency of flagellar movement could be regulated within the approximate range of 30–80 Hz by vibrating the micropipette holding the head of the demembranated sea urchin sperm (Gibbons et al., 1987; Eshel et al., 1990). These results implied that beat frequency is not determined only by ATP concentration. In the present study, we directly demonstrated that beat frequency is also determined by the 'movable length' of the flagellum as well as the ATP concentration. In one flagellum, the shorter the 'movable length', the higher becomes the beat frequency. Furthermore, the sliding velocity seemed to be approximately preserved during this procedure. Therefore, we concluded that ATP concentration determined the sliding velocity, not the beat frequency, which is probably determined by the sliding velocity of microtubules, the length of the flagellum and, presumably, some other external factor such as viscosity of the medium.

We express our thanks to Dr S. Baba at Ochanomizu University for supplying his flagellar movement auto-analyzing soft 'Bohboh'. This work was partly supported by Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (17049012).

References

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

Eshel, D., Shingyoji, C., Yoshimura, K., Gibbons, I. R. and Takahashi, K. (1990). Transient behavior of sea urchin sperm flagella following an abrupt change in beat frequency. *J. Exp. Biol.* **152**, 441-451.

Gibbons, I. R. (1974). Mechanisms of flagellar motility. In *The Functional Anatomy of the Spermatozoon* (ed. B. A. Afzelius), pp. 127-140. Oxford and New York: Pergamon.

Gibbons, I. R. (1975). The molecular bases of flagellar motility in sea urchin spermatozoa. In *Molecules and Cell Movement* (ed. S. Inoue and R. E. Stephens), pp. 207-232. New York: Raven Press.

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

Goldstein, S. F. (1969). Irradiation of sperm tails by laser micro beam. *J. Exp. Biol.* **51**, 431-441.

Hiratsuka, T. (1999). ATP-induced opposite changes in the local environments around Cys (697) (SH2) and Cys (707) (SH1) of the myosin motor domain revealed by the prodan fluorescence. *J. Biol. Chem.* **274**, 29156-29163.

Inaba, K. (2003). Molecular architecture of the sperm flagella: molecules for motility and signaling. *Zool. Sci.* **20**, 1043-1056.

Ishiguro, K., Murofushi, H. and Sakai, H. (1982). Evidence that cAMP-dependant protein kinase and a protein factor are involved in reactivation of Triton X-100 models of sea urchin and starfish spermatozoa. *J. Cell Biol.* **92**, 777-782.

Lindemann, C. B. (1994). A model of flagellar and ciliary functioning which uses the forces transverse to the axoneme as the regulator of dynein activation. *Cell Motil. Cytoskel.* **29**, 141-154.

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.

Okuno, M. (1980). Inhibition and relaxation of sea urchin sperm flagella by vanadate. *J. Cell Biol.* **85**, 712-725.

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.

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

Okuno, M. and Morisawa, M. (1982). Cyclic AMP and initiation of flagellar movement in rainbow trout spermatozoa. In *Biological Functions of Microtubules and Related Structures* (ed. H. Sakai, H. Mohri and G. G. Boricy), pp. 151-162. New York: Academic Press.

Shingyoji, C. and Takahashi, K. (1995). Cyclical bending movements induced locally by successive iontophoretic application of ATP to an elastase-treated flagellar axoneme. *J. Cell. Sci.* **108**, 1359-1369.

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

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.

Walker, P. J. (1961). Organization of function in trypanosome flagella. *Nature* **189**, 1017-1018.

Weber, G. and Farris, F. J. (1979). Synthesis and spectral properties of a hydrophobic fluorescent probe;6-propionil-2-(dimetylamino)naphthalene. *Biochemistry* **18**, 3075-3078.

Woolley, D. M. and Bozkurt, H. H. (1995). The distal sperm flagellum: its potential for motility after separation from the basal structures. *J. Exp. Biol.* **198**, 1469-1481.