Rotating the plane of imposed vibration can rotate the plane of flagellar beating in sea-urchin sperm without twisting the axoneme

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Summary

When the head of a sea-urchin sperm is held in the tip of a micropipette and vibrated laterally, the flagellum beats in phase with the imposed vibration. Rotation of the plane of pipette vibration around the head axis induces a corresponding rotation of the plane of beating, in both live and reactivated sperm. Detailed analysis of the waveforms occurring at different stages of this rotation shows that the characteristic asymmetry of the flagellar bending waves rotates along with the plane of beat. The positions of small polystyrene beads attached as markers on the axonemes of demembranated sperm flagella appear unaffected by the rotation of the beat plane and asymmetry. The imposed rotation of the waveform is thus the result of a rotation of the coordinated pattern of sliding among the doublet tubules of the axoneme, and is not accompanied by a twisting of the whole axonemal structure. These data indicate that neither the plane of flagellar beat nor the direction of beat asymmetry is tightly dependent upon a structural or chemical specialization of particular members of the nine outer doublet microtubules, but that both are the result of some regulatory structure that can be forced to rotate relative to the outer structure of the axoneme.

Key words: flagella, wave asymmetry, sliding pattern.

Introduction

The beating of eukaryotic flagella and cilia is believed to be due primarily to the linear oscillatory sliding motion between the nine outer doublet microtubules of the axoneme (Gibbons, 1981; Shingyoji et al. 1977; Takahashi et al. 1982). In sea-urchin sperm, flagellar bends are normally formed and propagated in almost one plane that has a constant orientation relative to the flagellar axoneme (Sale, 1986). The bends on opposite sides of the flagellum usually differ in angle, with those on the side of greater angle being known as principal bends and those on the side of lesser angle as reverse bends (Gibbons and Gibbons, 1972). This difference in bend angles gives an asymmetry to the flagellar beat pattern that makes the swimming sperm traverse a helical path in free fluid and a circular path when close to a glass slide (Gray, 1955). The degree of asymmetry in the flagellar beat pattern can be modulated by changing the concentration of free Ca$^{2+}$ (Brokaw, 1979), and changes in the degree of Ca-mediated asymmetry are believed to constitute the basis for sperm chemotaxis (Brokaw, 1987). However, the detailed mechanism by which Ca$^{2+}$ regulates asymmetry remains little known (Eshel and Brokaw, 1987).

We recently found that, in live and reactivated sea-urchin sperm flagella, the plane of beat can be forced to rotate relative to the sperm head by imposing a sinusoidal vibration of changing orientation on the sperm head (Gibbons et al. 1987; Katada et al. 1989). Several possible mechanisms for this imposed rotation of the plane of beat and its reversal can be envisaged. In the earlier study, we were able to exclude the possibility of the sperm head slipping relative to the micropipette by the fact that the flagellar beat plane spontaneously recovers back to its original orientation when the external vibration is terminated. However, we were unable to determine whether the basal region of the flagellar axoneme becomes twisted as the beat plane rotates or whether the axoneme remains untwisted and it is just the pattern of sliding among the doublets that rotates along with the plane of beat. In the former case the cylinder formed by the nine individual doublet microtubules, observed in the mid or distal region of the flagellum, would rotate relative to the sperm head, keeping a constant spatial relationship to the plane of beat, whereas in the latter case the cylinder of nine doublet microtubules would maintain a constant orientation and not rotate relative to the sperm head.

In this paper, we report observations made by placing beads as markers on the flagellar axoneme that show that no twisting of the whole axonemal structure accompanies the imposed rotation of the beat plane. This rotation of the beat plane must thus result from a rotation of the pattern of sliding among the doublet tubules of the axoneme. Additional new data show that the direction of waveform asymmetry rotates along with the beat plane, suggesting that the mechanism of asymmetry is closely associated
with that regulating the plane of beat. Kinetic studies on the spontaneous unwinding of the beat plane that follows forced winding are described in the accompanying paper (Takahashi et al. 1991).

Materials and methods

Conditions of flagellar motility

Live spermatozoa of the sea-urchin, *Hemicentrotus pulcherrimus*, were observed in Ca-free artificial sea water as described previously (Shingyoji et al. 1991). Demembranation was performed by incubating the sperm for 30–90 s in a medium containing 0.2 M potassium acetate, 2 mM MgSO4, 2 mM EGTA, 20 mM Tris–HCl (pH 8.2), 1 mM dithiotreitol, 0.04 % CHAPS (3-[3-cholamidopropyl] dimethylammoniom) 1-propane sulphonate) and 0.01 % Nonidet P-40 (Kajita et al. 1989). The demembranated sperm were then reactivated in a medium having the same composition as the demembranation medium except for the omission of the detergents and the addition of 2% (w/v) polyethylene glycol and 2 mM ATP. All experiments were performed at room temperature (21–24°C).

**Micropipette vibration**

The head of the spermatozoon being observed was held by gentle suction in the tip of a vibrating micropipette (Shingyoji et al. 1991). Under these conditions, the beat frequency of the sperm flagellum has a strong tendency to become synchronized with the frequency of micropipette vibration, while the plane of the flagellar beating becomes aligned with the plane of pipette vibration (Gibbons et al., 1987; Shingyoji et al. 1991). In the present experiments, the micropipette was made to oscillate in a plane by applying in-phase sinusoidal voltages, generated by a computer program, to two orthogonal piezoelectric bimorphs (Fig. 1) (Corey and Hudspeth, 1980; Gibbons et al. 1987; Eshel and Gibbons, 1989; Eshel et al. 1990; Shingyoji et al. 1991). The orientation of the vibration plane of the micropipette was determined by the relative amplitudes of the sinusoidal voltages applied to the piezomorphs. The plane of vibration could thus be made to rotate in either direction around the longitudinal axis of the pipette by appropriate changes in the relative amplitudes of these voltages.

The natural beat frequencies of live sperm (with no vibration) were about 45 Hz, while those of reactivated sperm were 35–40 Hz. For each experiment, the frequency of micropipette vibration was set approximately equal to the natural beat frequency, and the amplitude of vibration was 15–18 μm (peak-to-peak). Rotation of the plane of micropipette vibration was performed at about 0.2 revolution per second. All clockwise or counterclockwise directions of rotation are defined as those seen by an observer viewing along the sperm from its head toward the tip of the flagellum. The term lateral vibration will be used to designate vibration perpendicular to the longitudinal axis of the sperm head, within the focal plane of the microscope. Vertical vibration will refer to vibration perpendicular to the focal plane.

Because of the way in which sperm were caught while swimming over a coverslip, the natural flagellar beat plane of sperm in a micropipette usually coincided approximately with the focal plane of the microscope. Subsequent initiation of vibration in the lateral plane brought almost the whole flagellar waveform accurately into the plane of focus. In order to minimize hydrodynamic interactions of the flagellum with the microscope coverslip, the pipette was kept more than 50 μm from the nearest surface.

**Light microscopy and video recording**

The flagellar movement was observed using an inverted microscope with phase-contrast optics and xenon flash illumination as described previously (Shingyoji et al. 1991). The movement of the sperm flagellum and micropipette were recorded on video tape at 200 frames s⁻¹ with a high speed video system (nac MHS200), using flash illumination synchronized to the recording frequency. To analyze the flagellar waveform, traces of the spermatozoa and the micropipette tip were made by hand from the screen of a video monitor (Panasonic WV-5470) onto sheets of transparent film. Bend angles were determined from the angles of tangents drawn to the flagellar waveform on either side of the bend (Goldstein, 1976; Brokaw, 1979).

**Attachment of polystyrene beads**

When a suspension of polystyrene beads (0.4 μm diameter, Nihon Gosei Gomu Co. Ltd. ‘Immutab’) was added to sperm, some reactivated sperm flagella, the beads attached spontaneously to the axonemes, presumably as a result of charge interaction. In order to obtain maximum resolution, the movements of flagellar axonemes with attached beads were observed with dark-field optics (x40 objective) and photographed on 35 mm film, rather than with video microscopy.

**Results**

As reported previously (Gibbons et al. 1987), when the head of a live sperm is held with a vibrating micropipette and the plane of the vibration is gradually rotated the beat plane of the flagellum rotates along with the plane of the vibrating pipette. In the present experiments, we found that such rotation of the plane of vibration could force the flagellar beat plane to rotate along with it for at least nine complete revolutions. Beyond this point, the beat plane showed an increasing tendency to slip back one half-revolution or more when attempts were made to impose additional winding.

**Flagellar waveform during the rotation of its beat plane**

Fig. 2 shows the waveform of a typical flagellum during nine revolutions of imposed counterclockwise winding. After each complete revolution of winding, the flagellum continues to beat regularly with a waveform that is similar if not identical to the initial one. However, examination of the waveforms after odd numbers of half-revolutions shows that the lateral asymmetry of flagellar waveform reverses with every half-cycle of rotation. Thus, the principal bends, which lie toward the right side of the figure before rotation (0), lie toward the left after a half-cycle of rotation (0.5) and then revert to the right again after one complete revolution (1.0). A similar reversal occurs with each successive half-revolution.

Fig. 3 illustrates the manner in which the flagellar asymmetry, indicated as the ratio of angles of bends with...
Fig. 2. Superimposed tracings of waveforms during 9 cycles of counterclockwise rotation in a live spermatozoon. The number above each group of superimposed tracings indicates the number of completed revolutions. P and R denote the direction of principal and reverse bends of the flagellum, defined as the bends of greater and lesser angle, respectively. Note that the direction of the principal bend reverses with each half-cycle of rotation. The tracings in each set represent successive frames at 5-ms intervals.

their convex side toward the left to those of bends with their convex side toward the right, changes as the degree of winding is increased. The oscillation of this ratio above and below a value of unity with successive cycles of imposed winding indicates that the asymmetry of the flagellar bending waves reverses concomitant with each additional half-cycle of imposed beat plane rotation. Rotation of the vibration plane in the clockwise direction resulted in essentially the same behaviour. The reversal of flagellar asymmetry after each half-cycle of rotation strongly suggests that the direction of asymmetry in bending rotates together with the beat plane.

There was no change in the degree of asymmetry in the flagellar waveform with increasing cycles of complete rotation (n=0, 1, 2...9), and similarly there was no change in the degree of asymmetry after successive \( n + 0.5 \) cycles of rotation. However, comparison of the flagellar waveform after \( n \) cycles of rotation (termed the normal orientation) with that after \( n + 0.5 \) cycles of rotation (termed the reversed orientation) shows that the latter is not exactly the mirror image of the former.

This difference is quantitated in Fig. 4, which shows the angles of bends at different positions along the length of the flagellum in normal and in reversed orientation. When the flagellum is in its normal orientation (Fig. 4B), the angles of principal bends are larger than those of reverse bends throughout their propagation along the length, as in a freely swimming sperm (Gibbons and Gibbons, 1972). However, when the flagellum is in reverse orientation (Fig. 4A), the angles of principal and reverse bends are essentially equal when the bends are located \( \sim 10 \mu m \) from the base, and it is only when the bends pass into the distal half of the flagellum that the difference between the principal and reverse-bend angles appears. The essentially symmetrical waveforms in the proximal region of flagella in reversed orientation appear to be due mostly to a local increase in the angle of the reverse bends, while the angle of the principal bends is unaffected. The increased angle of the reverse bends in the proximal region of the flagellum is presumably a non-propagating effect of the forces imparted by the symmetrical vibration of the micropipette.

Essentially similar differences in waveforms in normal and reversed orientations were observed during several cycles of winding. We found that no change in asymmetry occurred with increasing amounts of rotation up to six revolutions in six different sperm. When the number of imposed revolutions was greater than six, however, the asymmetry measured at \( 20 \mu m \) often appeared to be slightly greater than the value obtained with fewer revolutions in the same sperm (Fig. 3).

Rotation of the flagellar beat plane

The flagellar beat plane of reactivated sperm that have been demembranated with CHAPS and Nonidet P-40, like that of live sperm, can be induced to rotate by imposed head vibration (Katada et al. 1989). Fig. 5 shows waveforms after successive half-cycles of counterclockwise beat-plane rotation. As in live sperm, the asymmetry of
Fig. 4. Changes of bend angles along length of the flagellum in live sperm in (A) reversed orientation (2.5 counterclockwise revolutions), and (B) normal orientation (3 counterclockwise revolutions). Angles of principal bends (●) are larger than those of reverse bends (○), except in the basal 10 μm of flagella in the reversed orientation. Each point indicates the average and standard deviation of bend angles obtained from four different sperm.

Fig. 5. Superimposed tracings of a typical reactivated flagellum during lateral phases of 7 counterclockwise imposed revolutions. The ATP concentration was 2 mM and the original beat frequency of the reactivated sperm was 39.6 Hz before the onset of vibration. The frequency of vibration was 41 Hz. As in live sperm, the direction of the flagellar asymmetry rotated along with the beat plane. The tracings in each set represent successive frames at 5-ms intervals.

Discussion

The plane of beat in cilia and flagella in most organisms is believed to be correlated with the orientation of the axonemal structure (Gibbons, 1961; Tamm and Horridge, 1976; Sale, 1986; Mohri et al. 1987). In the present study, the flagellar beat plane was shifted from its natural orientation by rotating the plane of external vibration applied to the sperm head. The strong tendency for the plane of flagellar beat to become aligned with the plane of

that of the axoneme, attachment of one or two beads did not appear to affect the flagellar beating (Brokaw, 1989). Fig. 6 shows four typical flagella beating laterally initially, vertically after 0.25 revolution and laterally again with reversed asymmetry after 0.5 revolution. In all cases, it can be seen that the position of a bead on an axoneme remains constant and appears unaffected by the rotation of the beat plane. The same result was obtained whether the bead was attached to the proximal, middle or distal region of the flagellum, although beads attached to the distal regions were difficult to photograph because they did not remain precisely within the same plane of focus. Similar results were obtained after either clockwise or counterclockwise rotation. These observations indicate that the axonemal framework of nine outer doublet microtubules does not twist relative to the micropipette during the rotation of the beat plane.
Fig. 6. Photographs of beating demembranated flagella to which a polystyrene bead (0.4 μm in diameter) has been attached, taken after rotation of 90° and 180° of the beat plane. The relative position of the bead on each of the flagellar axonemes does not change upon the rotation of the beat plane, although the flagellar asymmetry reverses as the winding proceeds from 0° to 180°. The results of four different reactivated sperm rotated counterclockwise are shown (A–D). The images of the beads appear artificially enlarged due to halation with dark-field illumination.
micropipette vibration presumably results from hydrodynamic forces, analogous to those responsible for the synchronization of bacterial flagella (Machin, 1963), that make it energetically more favourable for the plane of flagellar beating to coincide with the plane of micropipette vibration. This result indicates that the beat plane of sea-urchin sperm flagella has an unexpectedly high degree of plasticity relative to the structural organization of the flagellar axoneme.

We can think of three general mechanisms by which external forces could, in principle, produce rotation of the flagellar beat plane. One possibility might be for it to result from passive slippage either between the sperm head and the micropipette or between the membrane and the sperm head with its attached axoneme. However, this possibility is excluded by the strong tendency for the natural orientation of the beat plane to restore itself spontaneously when the external vibration is terminated (Gibbons et al. 1987; Shingyoji et al. 1991), as well as by the reversal of asymmetry with each half-turn of rotation. Moreover, the similar responses of both live and demembranated sperm (Takahashi et al. 1991) indicate that the plasma membrane is not involved in the rotation mechanism. A second possibility would be for the entire 9+2 axonemal structure to become twisted in its basal region, so that most of the length of the axoneme would rotate relative to the pipette and the head. This possibility, which would in any event make it difficult to explain the occurrence of as many as nine complete revolutions of the beat plane without irreversible physical damage, is excluded by the present observation indicating that a bead attached to one side of a demembranated axoneme appears attached to the same side of the axoneme after a 90° or a 180° rotation of the beat plane. It is highly improbable that the attachment site of the bead would move continuously on the axoneme in the direction opposite to that of the imposed rotation. The third possibility is that the rotation of the beat plane and asymmetry are the result of a rotation in the coordinated pattern of active sliding among the nine outer doublets of the axoneme, without any rotation of the sperm as a whole or any twisting of its axoneme. This pattern of sliding underlies the formation and propagation of planar bending waves in the sperm flagellum, so that rotation in the pattern of sliding would result in rotation of the beat plane. In the absence of other possibilities, we conclude that such a rotation of the pattern of sliding among the outer doublets provides the only possible mechanism for the observed rotation of the beat plane and asymmetry.

This conclusion implies that it is possible for flagellar beating to occur with the plane of bending in any orientation relative to the axonemal framework of nine outer doublet tubules. As far as it is known, the nine outer doublets and the dynein arms on them are chemically and structurally equivalent, except for the seemingly attached ‘bridge’ between doublets 5 and 6 (Afzelius, 1959). Some previous reports have suggested that there are functional differences in the sliding and bending ability of particular doublets. For example, a characteristic pattern of axonemal disintegration into two sub-groups that always contain the same doublets has been observed after extraction with ATP in sperm flagella of rat (Olson and Linck, 1977) and sea-urchin (Sele, 1986). The switch-point hypothesis (Satir, 1985) that explains some of the oscillatory properties of flagellar beating also predicts distinct functionality of the different doublet microtubules. However, these apparent differences may be due to the influence of the regulatory mechanisms that normally coordinate the activity of the doublets, rather than to inherent differences in the doublets themselves. Our findings strongly suggest that the nine outer doublets are potentially equivalent in their ability to originate and propagate bends in any plane. Since we found no discontinuity or abnormal phase within a rotation cycle, we can only speculate about whether active sliding can occur between the bridged doublets, 5 and 6. It is not possible, then, that this sliding might occur between doublets 6 and 7 or between doublets 4 and 5 in order to maintain smooth rotation of the beat plane during all the rotation cycle phases. The hypothesis of the nine doublets being functionally equivalent is supported by the observations showing equivalent sliding ability during the disintegration of trypsin-treated axonemes (Takahashi et al. 1982; Yano and Miki-Noumura, 1980).

The mechanism that coordinates the sliding activity of the outer doublet microtubules to produce planar bending waves is not known in detail. Results from a variety of organisms have suggested that the central pair of microtubules is involved in determining the planarity of beating (Gibbons, 1961; Sale, 1986; Tamm and Horridge, 1970; Mohri et al. 1987; Brokaw and Luck, 1985; Baccetti et al. 1979; Gibbons et al. 1985; Ishijima et al. 1988). Moreover, in some cilia that beat with a three-dimensional waveform, it has been suggested that the central pair rotates continuously within the axoneme during normal beating (Omoto and Kung, 1980). Other experimental results also have suggested that the central pair may play a role in determining the asymmetry of the flagellar waveform (Brokaw et al. 1982; Brokaw and Luck, 1985; Gibbons et al. 1985). Our finding that the asymmetry of the flagellar waveform appears tightly associated with the beat plane and rotates along with it supports the above hypotheses and suggests that the orientation of the central pair is not only correlated with the beat plane, but most probably determines the beat plane and the asymmetry. The coupling between the flagellar asymmetry and the orientation of the central pair might be the result of a Ca-induced curvature in the central pair that rotates along with it and serves as the biased baseline that appears to underlie the asymmetry of flagellar beating (Éshel and Brokaw, 1987).

If the central pair of tubules regulates the asymmetry and plane of flagellar beating, it presumably coordinates dynein arm activity through the central sheath/radial spoke complex that connects to each of the nine outer doublets. This general hypothesis receives support from the fact that the motility of paralyzed mutant flagella of Chlamydomonas that lack the central pair and radial spokes can be restored by an extragenic suppressor mutation that by-passes the need for the missing structures through a compensating alteration in one of the motor subunits of the dynein arms (Huang et al. 1982; Brokaw and Luck, 1985). However, the evidence for this regulatory activity is wholly indirect at present. Future genetic and ultrastructural studies of the interpretation of the central pair to the components of the outer doublet tubules will be necessary to shed more direct light on the details of this putative regulatory mechanism.

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