CELL STRUCTURE AND FUNCTION 24: 43–54 (1999)

Effects of the Central Pair Apparatus on Microtubule Sliding Velocity in Sea Urchin Sperm Flagella

Misako Yoshimura and Chikako Shingyoji*

Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Tokyo 113-0033, Japan

ABSTRACT. **To produce oscillatory bending movement in cilia and flagella, the activity of dynein arms must be regulated. The central-pair microtubules, located at the centre of the axoneme, are often thought to be involved in the regulation, but this has not been demonstrated definitively. In order to determine whether the central-pair apparatus are directly involved in the regulation of the dynein arm activity, we analyzed the movement of singlet microtubules that were brought into contact with dynein arms on bundles of doublets obtained by sliding disintegration of elastase-treated flagellar axonemes. An advantage of this new assay system was that we could distinguish the bundles that contained the central pair apparatus from those that did not, the former being clearly thicker than the latter. We found that microtubule sliding occurred along both the thinner and the thicker bundles, but its velocity differed between the two kinds of bundles in an ATP concentration dependent manner. At high ATP concentrations, such as 0.1 and 1 mM, the sliding velocity on the thinner bundles was significantly higher than that on the thicker bundles, while at lower ATP concentrations the sliding velocity did not change between the thinner and the thicker bundles. We observed similar bundle width-related differences in sliding velocity after removal of the outer arms. These results provide first evidence suggesting that the central pair and its associated structures may directly regulate the activity of the inner (and probably also the outer) arm dynein.**

Key words: dynein/flagella/microtubule/central pair/sperm/sea urchin

The aim of this study is to test the hypothesis that the motile activity of dynein in the flagellar axoneme is regulated by the central-pair microtubules. This hypothesis is based on previous studies indicating that the central pair/radial spoke complex may be involved in the rotation as well as determination of the flagellar beat plane, and the oscillatory bending. Thus, in the sea urchin and other animals, the central pair has been suggested to determine the planarity of flagellar (or ciliary) beating (4, 6, 7, 35, 47). Rotation of the central pair has been found in *Paramecium, Micromonas*, and *Chlamydomonas* (13, 19, 26, 27, 28). The rotatability of the beating plane in sea urchin sperm flagella under imposed head vibration is thought to be related to the rotation of the central pair microtubules, and this probably supports the idea that the central pair microtubules may possibly be involved in determining the beating plane (8, 39). It is thought that as the central pair rotates, a signal regulating the activity of dynein is transmitted from the central pair to the dynein arms via the radial spokes. In *Chlamydomonas*, mutants lacking the central pair or the radial spokes have paralyzed flagella, although their dynein arms are normal and can generate sliding of outer doublets in enzyme-treated axonemes (44, 51). The analysis of suppressor mutations which restore beating to the paralyzed mutants without restoring missing structures (central pair or radial spokes) has indicated that the central pair regulates microtubule sliding in a pathway involving the radial spokes (3, 15, 30, 31, 32, 33).

The role of the radial spoke in the regulation of phosphorylation of the inner dynein arms, which may be related to the control of dynein-driven microtubule sliding (a decrease in the sliding velocity), has been shown by using radial spoke-deficient mutants of

* To whom correspondence should be addressed:
Tel: +81–3–5841–4065, Fax: +81–3–5841–4385
E-mail: chikako@biol.s.u-tokyo.ac.jp

Abbreviations: EGTA, ethylene glycolbis(2-aminoethyl ether)N,N,N′,N′-tetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid.

Chlamydomonas (9, 14, 44). The central pair may regulate the radial spokes. To generate normal oscillatory bending movements, in intact axonemes, both the central pair and the radial spokes must always be functional in regulating the activity of dynein. This, however, cannot be confirmed in beating flagella, because it is difficult to evaluate the activity of dynein in the presence and absence of the signal from the central pair apparatus. One way to overcome this difficulty is to measure the sliding activity of the dynein arms on the doublets in the presence and in the absence of the central pair under conditions that preserve the regulatory mechanisms of the axoneme as much as possible. In the conventional procedure to analyze the sliding activity, axonemes are treated with an enzyme, such as trypsin (45). Trypsin-treated axonemes, however, are incapable of bend formation and oscillation, which indicates that the mechanism regulating the activity of dynein is not preserved (40). We have previously shown that elastase-treated flagellar axonemes of the sea urchin sperm are still capable of bend formation and oscillation when reactivated locally by ATP-iontophoresis (40). Furthermore, the elastase-treated axonemes slide apart into a pair of thinner and thicker bundles of doublets in the presence of 1 mM ATP and 10^{-4} M Ca^{2+} and only the thicker bundles contain the central pair microtubules (Shingyoji *et al.*, in preparation). In this respect, these thinner and thicker bundles are uniquely suited for the study of the regulation of the activity of dynein by the central pair.

In this study, we developed a new assay system for microtubule sliding by modifying previous methods in which singlet microtubules were made to interact with dynein arms on isolated doublets (42, 52). In our new assay system, we used, instead of individual doublets, doublet bundles with or without the associated central pair microtubules. By adding singlet microtubules to these bundles and analyzing their sliding movement caused by the activity of the dynein arms on the bundles, we found that the presence or absence of the central pair significantly affected both the frequency of occurrence and the velocity of sliding movement. This effect of the central pair on the sliding velocity was observed in the bundles from which the outer arms had been removed as well as in the bundles in which both the outer and the inner arms were intact. In either case, however, the effect of the central pair was observed only at high ATP concentrations. Our results show for the first time that the sliding activity of the inner (and probably also the outer) dynein arms are directly regulated by the central pair apparatus.

Materials and Methods

Axonemes and microtubules

Sperm of the sea urchin *Pseudocentrotus depressus* and *Clypeaster japonicus* were suspended in the same volume of Ca^{2+}-free artificial sea water (465 mM NaCl, 10 mM KCl, 25 mM $MgSO_4$, 25 mM $MgCl_2$, and 2 mM Tris-HCl, pH 8.0) and demembranated with 15 volumes of demembranating solution containing 0.04% (w/v) Triton X-100, 0.15 M K-acetate, 2 mM $MgSO_4$, 2 mM EGTA, 1 mM dithiothreitol (DTT), 10 mM Tris-HCl, pH 8.0 for 45 seconds at 20–23°C (*Pseudocentrotus*) or 24–27°C (*Clypeaster*). This suspension was diluted with 16 volumes of reactivating solution (Tris) containing 0.15 M K-acetate, 2 mM $MgSO_4$, 2 mM EGTA, 1 mM DTT, 2% (w/v) polyethyleneglycol (MW 20,000) and 10 mM Tris-HCl, pH 8.0 without ATP. For *Clypeaster*, 20 mM HEPES (pH 7.8) was used instead of Tris (pH 8.0) in both demembranating and reactivating solutions. Preparations that showed less than 90% reactivation at 1 mM ATP were discarded.

The demembranated sperm suspension was pelleted at 11,000 g and labelled with 15–40 μM tetramethylrhodamine (Molecular Probes, C-1171) in reactivating solution (containing 20 mM HEPES instead of Tris; without ATP) for 4 min (on ice). The rhodamine-labelling was stopped by 0.1 M Tris-HCl (pH 8.0). The labelled sperm was pelleted at 17,000 g, resuspended in reactivating solution (HEPES; without ATP) and homogenized to obtain axonemal fragments. Sperm heads were removed by centrifugation at 2,000 g, the supernatant containing axonemal fragments was centrifuged at 17,000 g and resuspended in reactivating solution (HEPES; without ATP).

To remove outer arms, demembranated sperm were extracted with 60 volumes of 0.6 M KCl (in reactivating solution without ATP) for 4 min before labelling with rhodamine. The beat frequency in 1 mM ATP of the outer-arm-depleted sperm of *Pseudocentrotus depressus* and *Clypeaster japonicus* were about 50% lower (*Pseudocentrotus*, 13.5 ± 1.6 Hz, n=39; *Clypeaster*, 22.9 ± 3.9 Hz, n=12) than that of the intact sperm (*Pseudocentrotus*, 27.3 ± 2.8 Hz, n=54; *Clypeaster*, 39.4 ± 2.9 Hz, n=10), indicating $\geq 80\%$ extraction of the outer arms (5, 53).

Singlet microtubules were assembled by mixing bovine tu-

Fig. 1. Video images showing paired bundles and sliding of a microtubule. A: A pair of bundles indicated by a filled arrowhead consists of a thinner bundle (left) and a thicker bundle (right). B and C: A microtubule attached to the thinner of the paired bundles and slid along it towards the right at a speed of 1.4 μm/sec in 0.02 mM ATP. The interval between B and C was 5.7 sec. A white arrowhead indicates the leading end of the microtubule. Bar, 10 μm. D: Schematic diagram showing paired bundles consisting of a thinner and a thicker bundle. In this configuration microtubules are expected to interact with the dynein arms on the thinner bundle which is attached to the glass surface. The thicker bundle contains central pair microtubules.

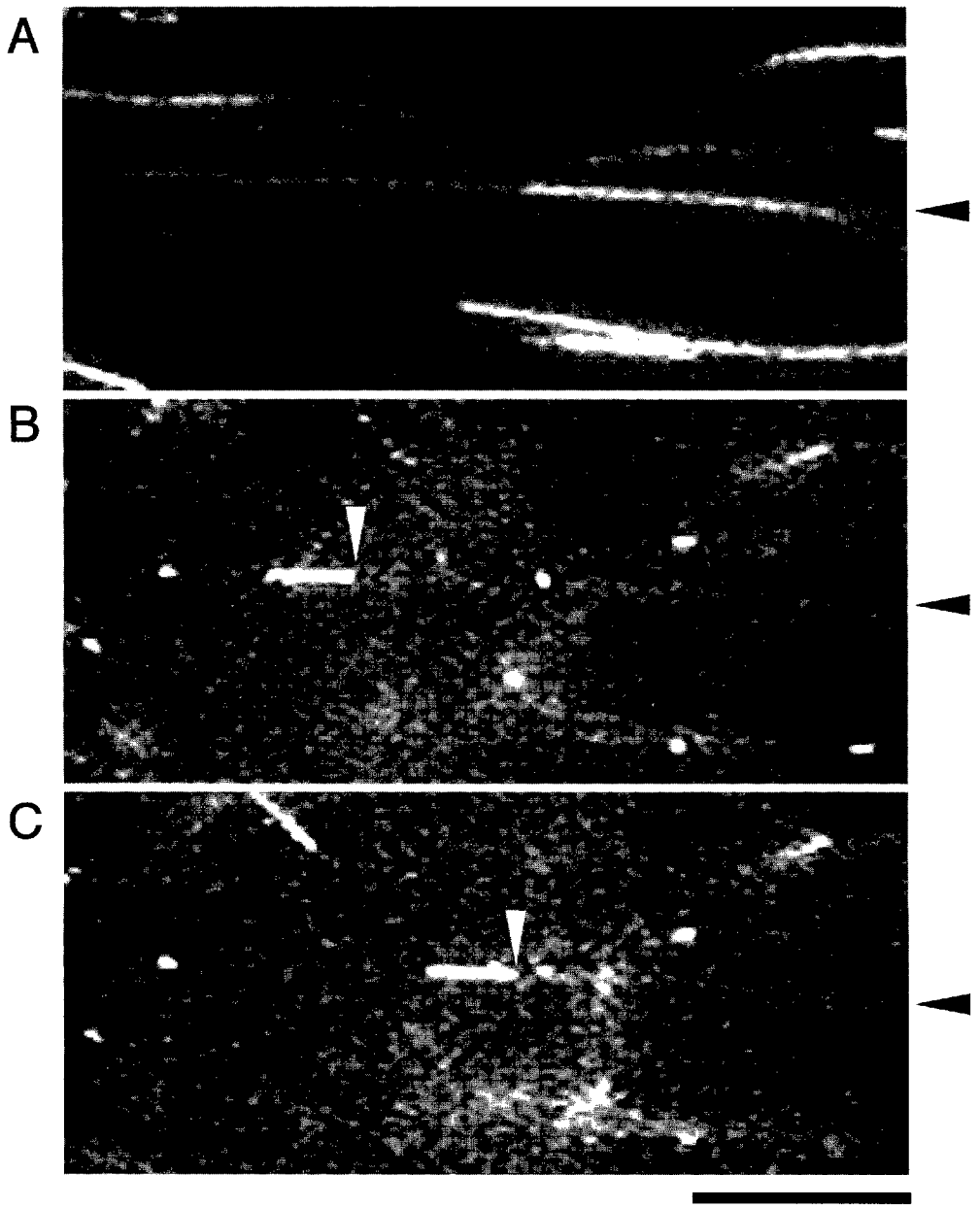

Fig. 1.

bulin and tetramethylrhodamine-labelled tubulin with GTP and were stabilized with 40–100 μM taxol. The mean length of the microtubules used was 2.1±2.0 μm (n=240; range, 0.4–16.4 μm). In order to distinguish rhodamine-labelled singlet microtubules from the disintegrated bundles of axonemal doublets which were also labelled with rhodamine, we used weakly labelled axonemes and brightly labelled microtubules.

Observation of microtubule sliding

We constructed a 5-μl chamber using a glass slide and a coverslip with 10 μm-thick polyester sheets as spacers. The space between the coverslip and the slide was sealed on two sides with enamel. A suspension of axonemal fragments was introduced into the chamber and treated with elastase (5 μg/ml elastase, Sigma type III and 5 μg/ml trypsin inhibitor, Sigma type I-S, in the reactivating solution containing 10^{-4} M Ca^{2+} without ATP) for 1–1.5 min. The elastase treatment was stopped with ovoinhibitor (50 μg/ml ovoinhibitor, Sigma type IV-O, in reactivating solution containing 10^{-4} M Ca^{2+} without ATP), and followed by perfusion with casein (1 mg /ml in reactivating solution containing 10^{-4} M Ca^{2+} without ATP). The perfusion of reactivating solution containing 10^{-4} M Ca^{2+} and 1 mM ATP then induced sliding disintegration of the axonemal fragments into two parts. As a next step to reduce the Ca^{2+} concentration, first the assay buffer (see below) without EGTA and then the assay buffer containing various concentrations of ATP were perfused. The microtubules were suspended in the assay buffer to which 2% (v/v) β-mercaptoethanol, 40 mM glucose, 430 μg/ml glucose oxidase, 70 μg/ml catalase, 10 μM taxol and various concentrations of ATP had been added. The assay buffer contained 70 mM K-acetate, 5 mM Mg-acetate, 20 mM HEPES, 2 mM EGTA, 0.1 mM EDTA and 1 mM DTT, pH 7.8. Since previous findings suggest that certain aspects of flagellar movement may change depending on whether the ATP concentration is above or below about 0.1 mM (21, 41, 48), we used 0.02 and 0.05 mM as lower ATP and 0.1 and 1 mM as higher ATP concentrations. The concentration of Ca^{2+} in the solution used for the observation of microtubule sliding was about 10^{-9} M (25).

Microtubule sliding was observed under a fluorescence microscope (Olympus, BX60) with a ×100 oil-immersion objective lens (Olympus PlanApo, NA=1.4) and recorded on video tape using a high-sensitivity silicon-intensified target camera (SIT camera, Hamamatsu Photonics, C2400-08) and a VHS video cassette recorder. To analyze the sliding velocity, the video images were traced by hand from the screen of a video monitor onto a sheet of transparent film. The sliding velocity was determined from the time measured by counting the number of video fields and the distance of microtubule movement measured on the traced images.

Results

Behaviour of microtubules on the doublet bundles

Application of 1 mM ATP to the elastase-treated axonemal fragments induced sliding disintegration which split each fragment longitudinally into two microtubule bundles of unequal thicknesses. These bundles will be referred to as the thicker and the thinner bundles. They were usually found in close pairs and were easily distinguished from one another under the fluorescence microscope (Fig. 1A). When a suspension of singlet microtubules was applied in the presence of ATP, some microtubules were observed to move along the bundles. Figure 1B and 1C show an example of a microtubule sliding on a thinner bundle. The movement was most probably caused by "exposed" dynein arms on one of the doublets in the bundle (Fig. 1D). Among the pairs of bundles, more than about 30% showed microtubule sliding along either the thinner or the thicker bundles (Table I). About half of the microtubules that came close to the bundles attached themselves to the bundles and showed either a unidirectional sliding movement (about 30%) or back-and-forth movements (about 20%) along the bundles. The remaining microtubules that came close to the bundles

Table I. NUMBER OF PAIRED BUNDLES THAT SHOWED MICROTUBULE SLIDING

	ATP concentration (mM)		Number of paired bundles	
			Total	Sliding observed
Outer and inner arms intact	High	1	282	103 (36%)
		0.1	54	33 (61%)
	Low	0.05	27	11 (41%)
		0.02	75	30 (40%)
Outer arms removed	High	1	167	52 (31%)
		0.1	54	21 (39%)
	Low	0.05	69	16 (23%)
		0.02	79	32 (41%)

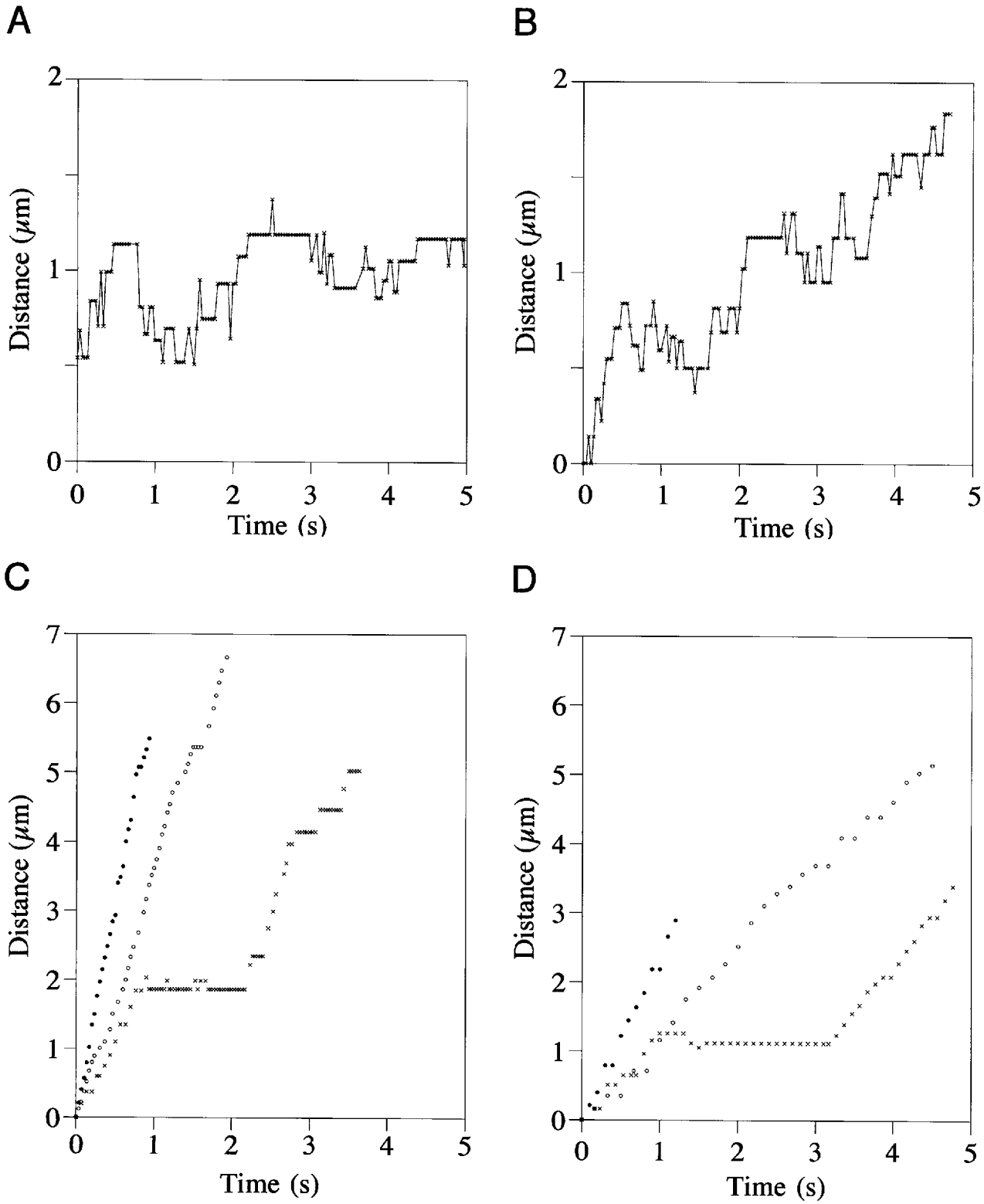

Fig. 2. Time courses of microtubule movement at 1 mM (A, C) and 0.02 mM (B, D) ATP. A and B show back-and-forth motion of microtubules. C and D each show three examples of microtubule sliding. Some microtubules showed smooth sliding (filled circles in C and D) while others showed interrupted sliding (open circles and crosses in C and D).

attached themselves to the bundles and remained stationary. These behaviours of the microtubule did not change when the ATP concentration was changed. Removal of the outer arms did not affect these characteristics of microtubule sliding along the bundles.

Figure 2 shows examples of back-and-forth (A, B) and sliding (C, D) movements of microtubules in the presence of 1 mM (A, C) and 0.02 mM (B, D) ATP. In the back-and-forth motion, the distance of each single movement was less than 0.5 μm. The frequency of the oscillation was a few Hz and did not depend on the ATP concentration. Apparently there was no regularity in the movement. The oscillating microtubules sometimes moved gradually towards one end of the bundle, as shown in Figure 2B (towards the upper direction in this figure). In contrast to the back-and-forth movement, the unidirectional sliding movement continued for several micrometres. We have previously confirmed the polarity of microtubule sliding in a similar axonemal preparation as used here (42), i.e., the movement of the sliding microtubules is always towards their plus ends, indicating that it is caused by dynein. Sometimes, the sliding of microtubules was interrupted by periods of pause (Fig. 2C, D). Both the number (from zero to several times) of pauses during a course of sliding movement and the duration (from about 30 msec to several sec) of each interruption were variable. Also there seemed to be no fixed region of the bundle where the interruption occurred. Some microtubules that were attached to the bundle and were stationary on the bundle began to move after several seconds of quiescence. Similar interruption of sliding was observed at different ATP concentrations and on both the thicker and thinner bundles. Thus, ATP concentration and the type of bundles did not qualitatively affect the basic features of microtubule behaviour on the bundles.

Effects of central pair and ATP concentration on the frequency of occurrence and the velocity of microtubule sliding

We found that both the frequency of occurrence and the velocity of sliding depend not only on the concentration of ATP but also on the type of the bundle. As described above, microtubule sliding occurred along

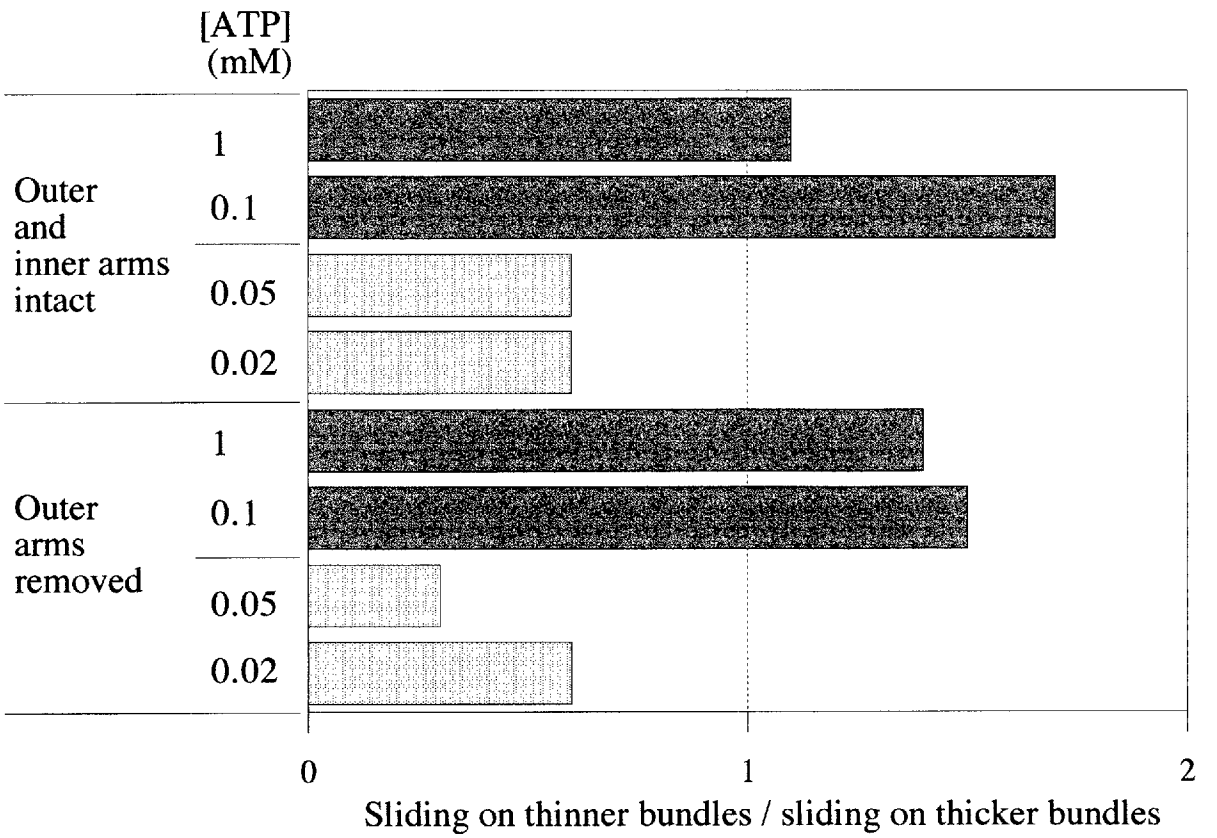

Fig. 3. Ratio of the number of occurrences of microtubule sliding observed on the thinner bundles to that on the thicker bundles. Upper columns and lower columns indicate the bundles with outer and inner dynein arms and the outer arm-depleted bundles, respectively.

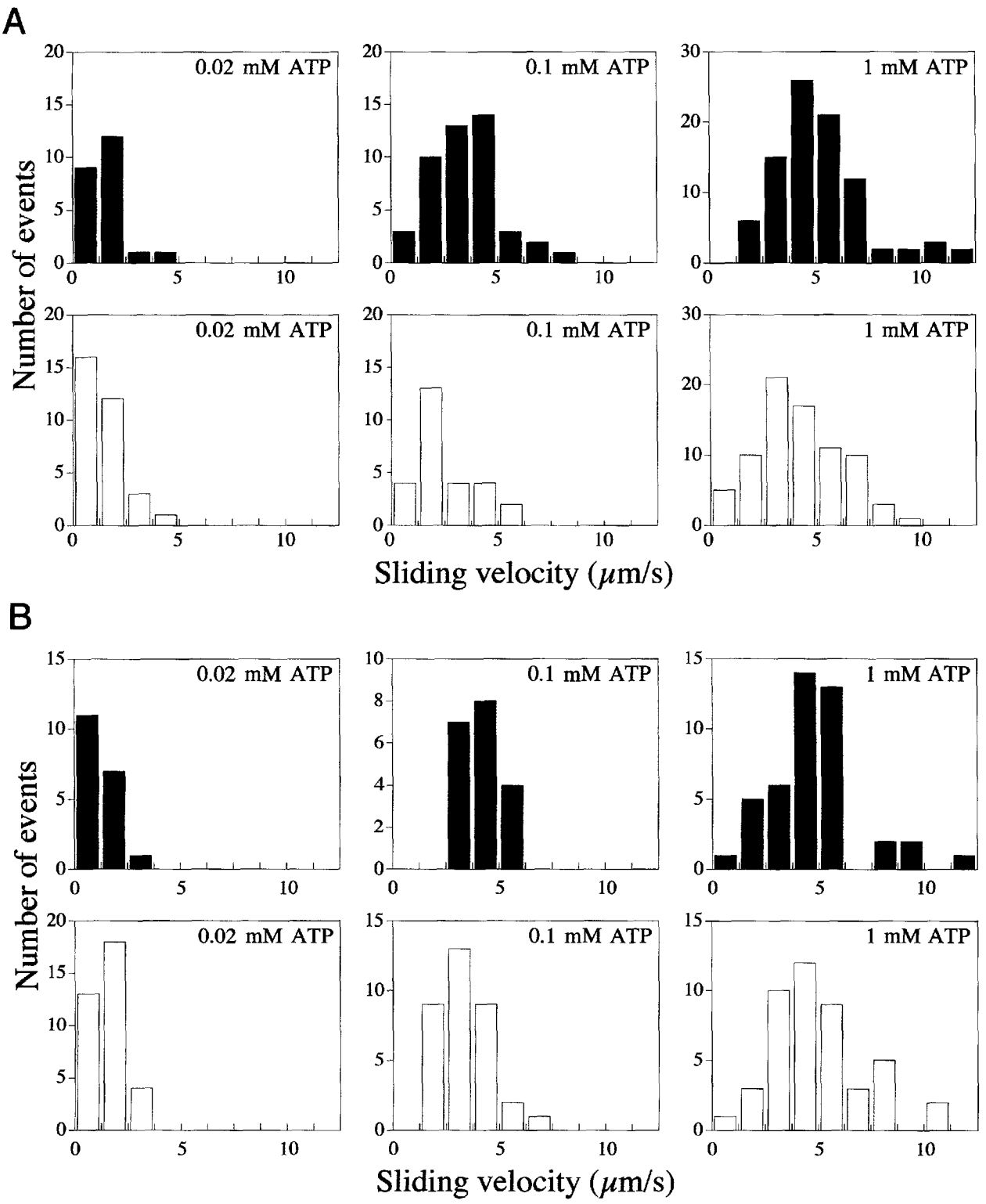

Fig. 4. Distribution of microtubule sliding velocities on thinner (filled bars) and thicker (open bars) bundles. A: sliding velocities in the doublet bundles with outer and inner dynein arms at 0.02, 0.1 and 1 mM ATP. B: sliding velocities in the outer arm-depleted bundles at 0.02, 0.1 and 1 mM ATP. At 0.02 mM ATP the sliding velocities on thinner and on thicker bundles were similar but at 0.1 mM and 1 mM ATP those on the thicker bundles were lower than those on the thinner bundles.

about 30% of the paired bundles examined. However, it did not take place equally on the thinner and thicker bundles. Figure 3 shows the ratio of the number of the thinner bundles on which microtubule sliding was observed to the number of the thicker bundles on which sliding was observed. It is interesting that the ratio was higher than 1 at high ATP concentrations and lower than 1 at low ATP concentrations. This indicates that sliding occurs more frequently on the thinner bundle at high ATP concentrations but on the thicker bundle at low ATP concentrations. This is also observed in the outer arm-depleted bundles.

In addition to the frequency of occurrence, the velocity of sliding was also affected by the ATP concentration and the type of the bundle. We determined the sliding velocity during smooth movements of microtubules (Fig. 2). The sliding velocities at any ATP concentration did not show a Gaussian distribution, but showed a broad distribution with a peak at around the middle and a longer tail towards higher sliding velocities (Fig. 4). Statistical differences between the sliding velocities on the thinner and the thicker bundles at 0.02 mM, 0.1 mM and 1 mM ATP in the doublet bundles with both the outer and the inner arms (Fig. 4A) and in the outer arm-depleted bundles (Fig. 4B) were examined by using Mann-Whitney U-test. We found that in either case the sliding velocity on the thinner bundles and that on the thicker bundles were not different at 0.02 mM ATP but were significantly different ($p<0.05$) at higher ATP concentrations (0.1 and 1 mM in the doublet bundles with outer and inner dynein arms, and 0.1 mM in the outer arm-depleted bundles). Figure 5 summarizes the mean sliding velocities for all conditions studied. We confirmed that the sliding velocities on the thinner and the thicker bundles were significantly different only at ATP concentrations higher than 0.1 mM (indicated with asterisks in Figure 5; since the distribution of the velocity was not Gaussian, standard deviation was not shown). Thus, at high ATP concentrations, the sliding velocity on the thicker bundles were significantly lower than that on the thinner bundles. These ATP-dependent changes in velocity as well as frequency of occurrence of sliding did not depend on the length of the microtubules interacting with the bun-

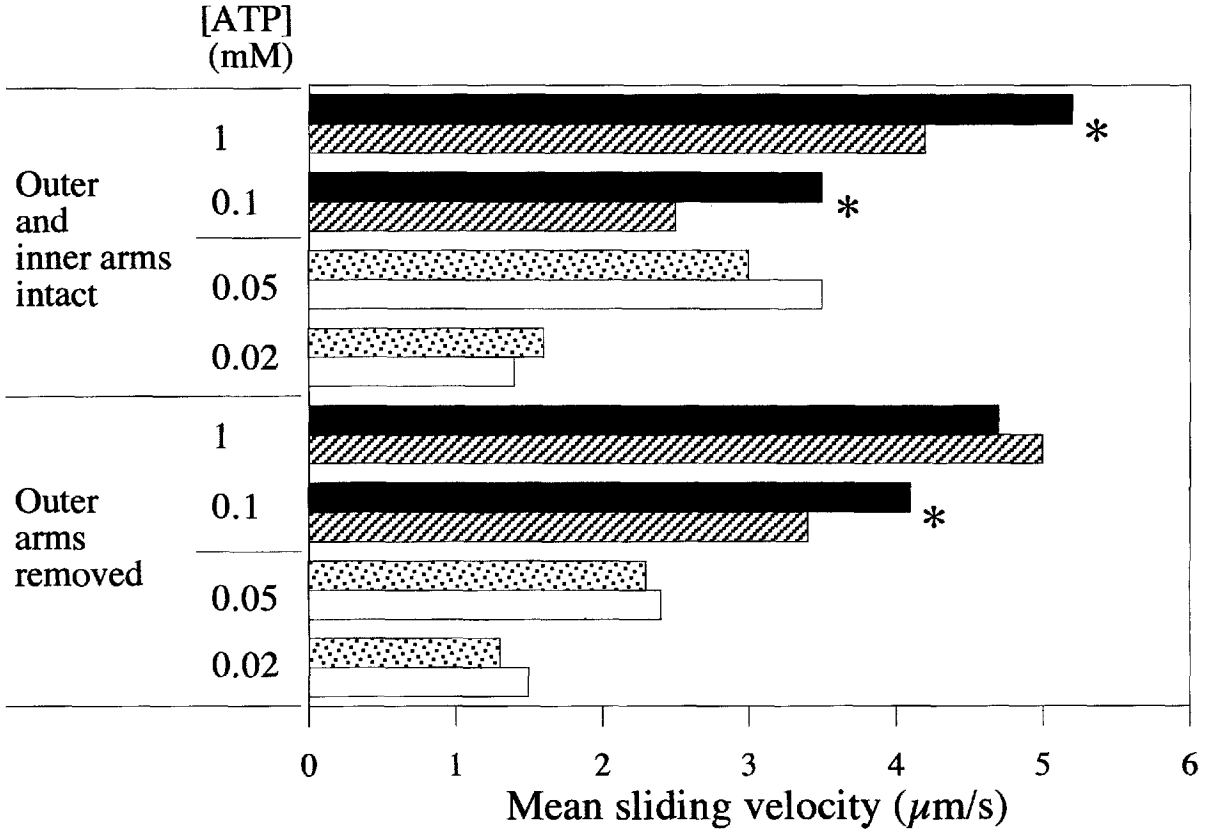

Fig. 5. Mean sliding velocity on thinner (filled bars and dotted bars) and thicker (hatched bars and open bars) bundles at various ATP concentrations. Asterisks indicate groups in which the sliding velocities on the thinner and those on the thicker bundles are significantly different by Mann-Whitney U-test ($p<0.05$). The number of paired bundles used for each condition is shown in Table I.

dles (data not shown).

In the doublet bundles with outer and inner dynein arms as well as in the outer arm-depleted bundles, the mean sliding velocity decreased with the decrease in ATP concentration (Figs. 4, 5). Unexpectedly, the sliding velocities were not changed by the removal of the outer arms. At any ATP concentration the mean sliding velocities in the bundles with outer and inner arms and in the outer arm-depleted bundles were similar, except at 0.05 mM ATP where there was a slight decrease.

Discussion

Previous studies have indicated that the central pair/radial spoke complex plays a role in the regulation of the activity of dynein arms in cilia and flagella. For example, many cilia and flagella beat in the plane perpendicular to the plane that includes the two microtubules of the central pair (4, 6, 7, 35, 47). Lack of the central pair or the radial spokes in *Chlamydomonas* paralyzes the flagella, even though the dynein arms of these mutants flagella are functional and can generate sliding disintegration of enzyme-treated axonemes (44, 51). In *Chlamydomonas* the radial spokes regulate the activity of dynein (probably of the inner arms) through suppression of a cAMP-mediated mechanism (9, 10, 11, 14, 44). Regulation of the activity of dynein arms by the central pair/radial spoke complex seems to be related to the ATP concentration. For example, axonemes isolated from radial-spoke or central-pair defective mutants of *Chlamydomonas* could be reactivated in low ATP concentrations ($\leq$0.05 mM) although they were paralyzed in high ATP concentrations ($\geq$0.1 mM) (29). The central pair apparatus is supposed to be a kind of "distributor" to regulate the activity of dynein in the axoneme, but this has not been directly shown.

The present study of microtubule sliding in the presence and absence of a central pair apparatus provides first experimental evidence that the central pair may directly regulate the activity of dynein arms to modify both the probability of occurrence and the velocity of microtubule sliding. Our results indicate that the central pair apparatus suppresses the dynein-driven sliding activity. Furthermore, they implicate the inner arms as a regulatory component, which directly receives a signal from the central pair through radial spokes. Several lines of evidence support this conclusion. First, sliding was observed less frequently in the presence than in the absence of the central pair. Second, the sliding velocity was significantly lower in the presence than in the absence of the central pair. Third, these effects of the central pair were observed only at ATP concentrations higher than 0.1 mM while at lower ATP concentrations neither the occurrence of sliding nor the sliding velocity was affected by the presence of the central pair. Finally, the removal of outer arms did not affect these central pair-associated changes. The dependence on the ATP concentration of the effects of the central pair apparatus strongly indicates that the present findings reflect the regulation of the activity of dynein *in vivo*. It is interesting that certain other properties of dynein, as well as the pattern of ATP-driven sliding disintegration of the axoneme, depend on whether the ATP concentration is above or below approximately 0.1 mM (21, 29, 41, 48). It is possible that different mechanisms regulate the activity of dynein in the two ATP concentration regimes.

The separation of the axonemal fragments into a pair of thinner and thicker bundles was unique to the elastase-treated sea urchin sperm flagellar axonemes. We have shown by electron microscopy that the thinner bundle consists of three (or four) doublets without the central pair microtubules and the thicker bundle consists of six (or five) doublets with the central pair microtubules (Shingyoji *et al.*, in preparation). The central pair is present only in the thicker bundles, while the radial spokes were present on all doublets in both of the bundles. Thus, the central pair and not the radial spokes must be responsible for the low frequency of observed sliding and the low sliding velocity in the thicker bundles. The central pair apparatus could act as a structural obstacle to microtubule sliding on the inner dynein arms of the bundle. But this is rather unlikely to occur because the shortest distance between the central pair and the inner dynein arms is twice as large as the diameter of a singlet microtubule (25 nm). Thus, the observed effect of the central pair is probably not an artifact caused by the preparation of the bundle but a genuine representation of the regulation of the activity of dynein.

Presumably the regulatory signal from the central pair apparatus is mediated by the radial spokes. In *Chlamydomonas*, it is suggested that the radial spokes activate the sliding activity of the inner dynein arms by dephosphorylation of one of the intermediate chains of the inner arm (9, 10, 11). The motility of *Chlamydomonas* flagella is inhibited by cAMP-dependent phosphorylation (12). In contrast, in sea urchin sperm the motility of demembranated flagella is activated by phosphorylation, and inhibited by dephosphorylation, of certain protein components (16, 24, 46). At the present time, we cannot explain these different responses in the two species. Our present results using the doublet bundles with and without the central pair microtubules suggest that this preparation may also be useful in elucidating the role of the radial spokes in the mechanism regulating the activity of dynein, particularly the functional relationship between the central pair and the radial spokes.

There has been no *in vitro* study of microtubule sliding along both the rows of outer and inner dynein arms. Although experiments have been carried out using a single doublet (42, 52), microtubules may not be able to interact with both the outer and the inner arms of the single doublets in these experiments since the dynein arms are not always standing upright. However, since we used bundles of more than three doublets in the present study, dynein arms were exposed along the doublet that formed one of the two edges of the bundle. They were thus pointing upwards in most cases so that a microtubule was able to interact with both rows of dynein arms. There are three possible ways in which the two rows of dynein arms are involved in the microtubule sliding: 1) both the outer and the inner arms interact simultaneously with the microtubule, 2) the outer and the inner arms interact with the microtubules independently, 3) either the outer or the inner arms interact with the microtubule. The distribution of sliding velocities in doublet bundles having both outer and inner arms was not Gaussian but showed a peak with a long tail. This result seems to be consistent with two of the possibilities: namely, both the outer and the inner arms may be able to interact simultaneously and cooperatively with a microtubule, or, alternatively, the outer and the inner arms may move the microtubules independently but at a similar velocity. Since the distribution of sliding velocity in the outer arm-depleted bundles was similar to that of the doublet bundles with both the outer and inner dynein arms (for example, at 1 mM ATP, the mean velocity was about 5 μm/sec), we cannot completely rule out the third possibility that only the inner arms were active in the doublet bundles with both the outer and inner arms. However, we should be cautious about such an argument based on the assumption of different sliding velocities between the outer and the inner dynein arms. The sliding velocities of one of the inner arms (C/A dynein, about 4 μm/sec) of the sea urchin sperm flagella determined by *in vitro* motility assay were similar to those of the outer arms (4–6 μm/sec) (22, 36, 54, 55). Inner and outer dyneins of other species, such as *Tetrahymena* (14S dynein and 22S dynein) and *Chlamydomonas* (I2 and I3, and $\alpha\beta$ subparticle of the outer dynein; subspecies of inner arm dynein) also showed similar sliding velocities (about 4–5 μm/sec at 1 mM ATP) in *in vitro* motility assay (18, 21, 34, 38, 43, 49). Thus, it is possible that the sliding speeds of singlet microtubules driven by the inner and the outer dynein arms are similar. The microtubule sliding velocity in *in vitro* motility assay is apparently very different from the sliding velocity in axonemal disintegration in the presence and absence of outer arms. Microtubule sliding during sliding disintegration of protease-treated axonemes with both the outer and inner dynein arms (mean velocity: 10–25 μm/sec) is twice to five times as fast as that of an outer arm-depleted axoneme (5–7 μm/sec) (20, 53). Also, the sliding velocity in disintegrating axoneme with both the outer and inner dynein arms (10–25 μm/sec) has been shown to be much higher than that measured in *in vitro* system (4–6 μm/sec). The reason for these differences is not clear. In the axoneme the dynein arms interact with the B-tubule of adjoining doublet microtubule, but in *in vitro* motility assay and also in our present assay system, singlet microtubules assembled from tubulin were used instead of doublets. Post-translational modification of tubulin of axonemal doublet microtubules has been known; in particular, polyglycylation and detyrosination of tubulin in B-tubule have been suggested to be involved in the regulation of flagellar motility in *Paramecium, Chlamydomonas* and sea urchin sperm (1, 2, 17, 23). The above difference in the sliding velocity may be caused by the difference in the microtubules. Using the present new assay system, quantitative analysis of the motile properties of outer and inner dynein arms and also the effects of different states of microtubules on the activity of dynein could be carried out in future.

The back-and-forth motion of microtubules we observed on the bundles is similar to the behaviour of microtubules observed by Vale *et al.* (1989) (50) in their *in vitro* assay of microtubule behaviour. This indicates that the oscillatory motion in the present preparation may also have been caused by thermal diffusion associated with a weak binding state of microtubule with dyneins. The interrupted sliding motion was clearly different from the back-and-forth motion. The interruption occurred randomly but the sliding motion between interruptions was always definitively clear and distinct from the back-and-forth motion. The interruption may have been caused by some damaged dynein arms. But the resumption of sliding after a certain period of pause, as well as the repetition of sliding and quiescence, may indicate cyclic activation of dynein arms on the doublets. If this is the case, the interrupted sliding may reflect the regulation of the activity of dynein.

The present finding of the effect of the central pair appartus on the activity of dynein is important for understanding the mechanism of flagellar beating. The regulation of sliding velocity by the central pair may be involved in the so-called switching mechanism in beating flagella (37). We expect that the present assay system using thinner and thicker bundles of doublets will be useful for further experiments to elucidate not only the roles of the regulatory components such as the central pair apparatus and radial spokes but also the nature of dynein itself.

Acknowledgments. We thank Prof. K. Takahashi (International Christian University) and Prof. R. Kamiya (University of Tokyo) for

discussion and critical reading of the manuscript, Drs. H. Higuchi (Tohoku University) and Y. Inoue (Osaka University) for suggestions about the preparation of microtubules and the present assay system. This work was supported by a Kurata Research Grant and a Grant from Nissan Science Foundation (to CS).

References

1. Bré, M.-H., Redeker, V., Quibell, M., Darmanaden-Delorme, J., Bressac, C., Cosson, J., Huitorel, P., Schmitter, J.-M., Rossier, J., Johnson, T., Adoutte, A., and Levilliers, N. 1996. Axonemal tubulin polyglycylation probed with two monoclonal antibodies: widespread evolutionary distribution, appearance during spermatozoan maturation and possible function in motility. *J. Cell Sci.*, **109**: 727–738.
2. Bré, M.-H., Redeker, V., Vinh, J., Rossier, J., and Levilliers, N. 1998. Tubulin polyglycylation: Differential post-translational modification of dynamic cytoplasmic and stable axonemal microtubules in *Paramecium*. *Mol. Biol. Cell*, **9**: 2655–2665.
3. Brokaw, C.J., Luck, D.J.L., and Huang, B. 1982. Analysis of the movement of *Chlamydomonas* flagella: the function of the radial-spoke system is revealed by comparison of wild-type and mutant flagella. *J. Cell Biol.*, **92**: 722–732.
4. Brokaw, C.J. and Luck, D.J.L. 1985. Bending patterns of *Chlamydomonas* flagella: III. A radial spoke head deficient mutant and a central pair deficient mutant. *Cell Motil.*, **5**: 195–208.
5. Gibbons, B.H. and Gibbons, I.R. 1973. The effect of partial extraction of dynein arms on the movement of reactivated sea-urchin sperm. *J. Cell Sci.*, **13**: 337–357.
6. Gibbons, B.H., Baccetti, B., and Gibbons, I.R. 1985. Live and reactivated motility in the 9+0 flagellum of *Anguilla* sperm. *Cell Motil.*, **5**: 333–350.
7. Gibbons, I.R. 1961. The relationship between the fine structure and direction of beat in gill cilia of a lamellibranch mollusc. *J. Biophys. Biochem. Cytol.*, **11**: 179–205.
8. 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.
9. Habermacher, G. and Sale, W.S. 1995. Regulation of dynein-driven microtubule sliding by an axonemal kinase and phosphatase in *Chlamydomonas* flagella. *Cell Motil. Cytoskel.*, **32**: 106–109.
10. Habermacher, G. and Sale, W.S. 1996. Regulation of flagellar dynein by an axonemal type-1 phosphatase in *Chlamydomonas*. *J. Cell Sci.*, **109**: 1899–1907.
11. Habermacher, G. and Sale, W.S. 1997. Regulation of flagellar dynein by phosphorylation of a 138-kD inner arm dynein intermediate chain. *J. Cell Biol.*, **136**: 167–176.
12. Hasegawa, E., Hayashi, H., Asakura, S., and Kamiya, R. 1987. Stimulation of in vitro motility of *Chlamydomonas* axonemes by inhibition of cAMP-dependent phosphorylation. *Cell Motil. Cytoskel.*, **8**: 302–311.
13. Hosokawa, Y. and Miki-Noumura, T. 1987. Bending motion of *Chlamydomonas* axonemes after extrusion of central-pair microtubules. *J. Cell Biol.*, **105**: 1297–1301.
14. Haward, D.R., Habermacher, G., Glass, D.B., Smith, E.F., and Sale, W.S. 1994. Regulation of *Chlamydomonas* flagellar dynein by an axonemal protein kinase. *J. Cell Biol.*, **127**: 1683–1692.
15. Huang, B., Ramanis, Z., and Luck, D.J.L. 1982. Suppressor mutations in *Chlamydomonas* reveal a regulatory mechanism for flagellar function. *Cell*, **28**: 115–124.
16. Ishiguro, K., Murofushi, H., and Sakai, H. 1982. Evidence that cAMP-dependent 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.
17. Johnson, K.A. 1998. The axonemal microtubules of the *Chlamydomonas* flagellum differ in tubulin isoform content. *J. Cell Sci.*, **111**: 313–320.
18. Kagami, O. and Kamiya, R. 1992. Translocation and rotation of microtubules caused by multiple species of *Chlamydomonas* inner-arm dynein. *J. Cell Sci.*, **103**: 653–664.
19. Kamiya, R. 1982. Extrusion and rotation of the central-pair microtubules in detergent treated *Chlamydomonas* flagella. *Cell Motil. Suppl.*, **1**: 169–173.
20. Kurimoto, E. and Kamiya, R. 1991. Microtubule sliding in flagellar axonemes of *Chlamydomonas* mutants missing inner- or outer-arm dynein: velocity measurements on new types of mutants by an improved method. *Cell Motil. Cytoskel.*, **19**: 275–281.
21. Mori, M. and Miki-Noumura, T. 1992. Inhibition of gliding movement by calcium in doublet microtubules on *Tetrahymena* ciliary dyneins *in vitro*. *Exp. Cell Res.*, **203**: 483–487.
22. Moss, A.G., Gatti, J.-L., and Witman, G.B. 1992. The motile β/IC1 subunit of sea urchin sperm outer arm dynein does not form a rigor bond. *J. Cell Biol.*, **118**: 1177–1188.
23. Multigner, L., Pignot-Paintrand, I., Saoudi, Y., Job, D., Plessmann, U., Rüdiger, M., and Weber, K. 1996. The A and B tubules of the outer doublets of sea urchin sperm axonemes are composed of different tubulin variants. *Biochemistry*, **35**: 10862–10871.
24. Murofushi, H., Ishiguro, K., Takahashi, D., Ikeda, J., and Sakai, H. 1986. Regulation of sperm flagellar movement by protein phosphorylation and dephosphorylation. *Cell Motil. Cytoskel.*, **6**: 83–88.
25. Okuno, M. and Brokaw, C.J. 1981. Calcium-induced change in form of demembranated sea urchin sperm flagella immobilized by vanadate. *Cell Motil.*, **1**: 349–362.
26. Omoto, C.K. and Kung, C. 1979. The pair of central tubules rotates during ciliary beat in *Paramecium*. *Nature*, **279**: 532–534.
27. Omoto, C.K. and Kung, C. 1980. Rotation and twist of the central-pair microtubules in the cilia of *Paramecium*. *J. Cell Biol.*, **87**: 33–46.
28. Omoto, C.K. and Witman, G.B. 1981. Functionally significant central-pair rotation in a primitive eukaryotic flagellum. *Nature*, **290**: 708–710.
29. 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.
30. Piperno, G., Mead, K., and Shestak, W. 1992. The inner dynein arms I2 interact with a "dynein regulatory complex" in *Chlamydomonas* flagella. *J. Cell Biol.*, **118**: 1455–1463.
31. Piperno, G., Mead, K., LeDizet, M., and Moscatelli, A. 1994. Mutations in the "dynein regulatory complex" alter the ATP-insensitive binding sites for inner arm dyneins in *Chlamydomonas* axonemes. *J. Cell Biol.*, **125**: 1109–1117.
32. Porter, M.E., Power, J., and Dutcher, S.K. 1992. Extragenic suppressors of paralyzed flagellar mutations in *Chlamydomonas reinhardtii* identify loci that alter the inner dynein arms. *J. Cell Biol.*, **118**: 1163–1176.
33. Porter, M.E., Knott, J.A., Gardner, L.C., Mitchell, D.R., and Dutcher, S.K. 1994. Mutations in the *SUP-PF-1* locus of *Chlamydomonas reinhardtii* identify a regulatory domain in the

β dynein heavy chain. *J. Cell Biol.*, **126**: 1495–1507.
34. SAKAKIBARA, H. and NAKAYAMA, H. 1998. Translocation of microtubules caused by the $\alpha\beta$, β and γ outer arm dynein subparticles of *Chlamydomonas*. *J. Cell Sci.*, **111**: 1155–1164.
35. SALE, W.S. 1986. The axonemal axis and Ca^{2+}-induced asymmetry of active microtubule sliding in sea urchin sperm tails. *J. Cell Biol.*, **102**: 2042–2052.
36. SALE, W.S. and FOX, L.A. 1988. Isolated β-heavy chain subunit of dynein translocates microtubules in vitro. *J. Cell Biol.*, **107**: 1793–1797.
37. SATIR, P. and MATSUOKA, T. 1989. Splitting the ciliary axoneme: implications for a "switch-point" model of dynein arm activity in ciliary motion. *Cell Motil. Cytoskel.*, **14**: 345–358.
38. SHIMIZU, T., FURUSAWA, K., OHASHI, S., TOYOSHIMA, Y.Y., OKUNO, M., MALIK, F., and VALE, R.D. 1991. Nucleotide specificity of the enzymatic and motile activities of dynein, kinesin, and heavy meromyosin. *J. Cell Biol.*, **112**: 1189–1197.
39. SHINGYOJI, C., KATADA, J., TAKAHASHI, K., and GIBBONS, I.R. 1991. Rotating the plane of imposed vibration can rotate the plane of flagellar beating in sea-urchin sperm without twisting the axoneme. *J. Cell Sci.*, **98**: 175–181.
40. 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.
41. 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.
42. SHINGYOJI, C., HIGUCHI, H., YOSHIMURA, M., KATAYAMA, E., and YANAGIDA, T. 1998. Dynein arms are oscillating force generators. *Nature*, **393**: 711–714.
43. SMITH, E.F. and SALE, W.S. 1991. Microtubule binding and translocation by inner dynein arm subtype I1. *Cell Motil. Cytoskel.*, **18**: 258–268.
44. SMITH, E.F. and SALE, W.S. 1992. Regulation of dynein-driven microtubule sliding by the radial spokes in flagella. *Science*, **257**: 1557–1559.
45. 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.
46. TAKAHASHI, D., MUROFUSHI, H., ISHIGURO, K., IKEDA, J., and SAKAI, H. 1985. Phosphoprotein phosphatase inhibits flagellar movement of Triton models of sea urchin spermatozoa. *Cell Struct. Funct.*, **10**: 327–337.
47. TAMM, S.L. and HORRIDGE, F.R.S. 1970. The relation between the orientation of the central fibrils and the direction of beat in cilia of *Opalina*. *Proc. Roy. Soc. London B*, **175**: 219–233.
48. TANAKA, M. and MIKI-NOUMURA, T. 1988. Stepwise sliding disintegration of *Tetrahymena* ciliary axonemes at higher concentrations of ATP. *Cell Motil. Cytoskel.*, **9**: 191–204.
49. VALE, R.D. and TOYOSHIMA, Y.Y. 1989. Microtubule translocation properties of intact and proteolytically digested dyneins from *Tetrahymena* cilia. *J. Cell Biol.*, **108**: 2327–2334.
50. VALE, R.D., SOLL, D.R., and GIBBONS, I.R. 1989. One-dimensional diffusion of microtubules bound to flagellar dynein. *Cell*, **59**: 915–925.
51. WITMAN, G.B., PLUMMER, J., and SANDER, G. 1978. *Chlamydomonas* flagellar mutants lacking radial spokes and central tubules: structure, composition and function of specific axonemal components. *J. Cell Biol.*, **76**: 729–747.
52. YAMADA, A., YAMAGA, T., SAKAKIBARA, H., NAKAYAMA, H., and OIWA, K. 1998. Unidirectional movement of fluorescent microtubules on rows of dynein arms of disintegrated axonemes. *J. Cell Sci.*, **111**: 93–98.
53. YANO, Y. and MIKI-NOUMURA, T. 1981. Recovery of sliding ability in arm-depleted flagellar axonemes after recombination with extracted dynein I. *J. Cell Sci.*, **48**: 223–239.
54. YOKOTA, E. and MABUCHI, I. 1994. Isolation and characterization of a novel dynein that contains C and A heavy chains from sea urchin sperm flagellar axonemes. *J. Cell Sci.*, **107**: 345–351.
55. YOKOTA, E. and MABUCHI, I. 1994. C/A dynein from sea urchin sperm flagellar axonemes: enzymatic properties and interaction with microtubules. *J. Cell Sci.*, **107**: 353–361.

(Received for publication, December 5, 1998
and in revised form, December 28, 1998)