Journal of Cell Science 104, 1251-1262 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Mechanical properties of ciliary axonemes and membranes as shown by paddle cilia

Michael Deiner, Sidney L. Tamm* and Signhild Tamm

Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543, USA

*Author for correspondence

SUMMARY

Cilia with a distal membrane expansion enclosing a coiled end of the axoneme (paddle cilia or discocilia) have been commonly reported in marine invertebrates. We recently showed that paddle cilia in molluscan veligers are artifacts of non-physiological conditions. Here we investigated the possible mechanisms of formation of paddle cilia under hypotonic conditions; particularly, whether a helical conformational change of doublet microtubules induced by Ca or proton flux is responsible. Typical paddle cilia are induced by hypotonic Ca-free solutions at normal or low pH, showing that axonemal coiling does not require Ca influx or proton efflux. In addition, Triton-demembranated straight axonemes do not coil in high Ca solutions. Most decisively, complete removal of paddle ciliary membranes with detergents, but not mere permeabilization, causes immediate uncoiling and straightening of the axonemes to approximately their original length before hypotonic treatment. These findings and other data show that axonemal coiling in paddles is due to membrane tensile stress acting on an elastic axoneme. Light and electron microscopy of paddles show that axonemes coil uniformly toward the direction of the effective stroke (doublets nos 5-6), even when beating is inhibited by sodium azide or glutaraldehyde before hypotonic treatment. This indicates that axonemes possess an intrinsic asymmetry of stiffness within the beat plane, independent of active microtubule sliding. Paddle cilia thus reveal important mechanical properties of ciliary axonemes and membranes that should be useful for understanding ciliary function.

Key words: axonemal coiling and elasticity, membrane tension, paddle cilia, molluscan veligers

INTRODUCTION

Cilia with a distal expansion of the membrane enclosing a curved or coiled end of the axoneme (paddle cilia or discocilia) have been widely reported in a variety of marine invertebrates. Various functions have been ascribed to paddle cilia, from acting as paddles to chemoreceptors (see Short and Tamm. 1991). Nevertheless, several authors have argued against the genuine nature of these organelles (Ehlers and Ehlers, 1978; Bone et al., 1982; Nielsen, 1987). Short and Tamm (1991) recently showed that paddle cilia in veliger larvae of bivalve molluscs are not genuine structures, but artifacts of hypo-osmotic solutions used by previous workers (Campos and Mann, 1988). Short and Tamm (1991) argued that most, if not all, examples of paddle cilia and discocilia are artifacts of non-physiological conditions. They proposed that coiling of the axoneme within the paddle is not due to tension by the surrounding membrane, but to a helical conformational change of the doublet microtubules induced by a Ca or proton flux across an osmotically stressed membrane.

We tested this model for paddle cilia formation in order to gain further knowledge of the structural and mechanical properties of ciliary axonemes and membranes. We found that axonemal coiling is due to tension exerted on an elastic axoneme by the enclosing membrane. Furthermore, the direction of coiling is specific, indicating an intrinsic asymmetry of stiffness of the axoneme within the ciliary beat plane. These findings illustrate important mechanical properties of ciliary axonemes and membranes that will be useful for understanding the mechanism and control of ciliary movement. Some of these findings have been published elsewhere in preliminary form (Deiner and Tamm, 1991; Deiner et al., 1992).

MATERIALS AND METHODS

Organisms

Stacks of *Crepidula fornicata* were collected near Woods Hole in summer, 1991, and maintained in running sea-water tables in the laboratory. *Crepidula* was advantageous for this study because the larval period is long, and thousands of fully formed, actively moving veligers are retained in egg capsules by females during the breeding season (Conklin, 1897). Egg capsules, each containing several hundred veligers, were removed from a female and torn open with forceps in a small dish of sea water to release the swimming larvae.

Two- to three-day-old veligers of *Mulinia lateralis* were obtained by fertilizing eggs and allowing embryos to develop in large bowls of sea water.

Before using, veligers of *Crepidula* or *Mulinia* were gently concentrated by low-speed centrifugation in the stems of 100 ml oil tubes.

Perfusion slides

Concentrated, swimming veligers were pipetted onto the center of a microscope slide ringed with vaseline. A 22 mm × 22 mm coverslip was placed over the center of the well and gently compressed to immobilize the larvae. Solutions were perfused under the coverslip by adding fluid to one of the open sides of the coverslip and removing excess solution from the other side.

Solutions

Different isotonic solutions (and their abbreviations) consisted of: normal sea water (SW); MBL artificial sea water, pH 8.3 (ASW); MBL Ca-free artificial sea water with 1 mM EGTA, pH 8.3 (CaF ASW); 1 M sorbitol, pH 3.7; 1 M sorbitol, 20 mM Tris, pH 7.0; 1 M sorbitol, 1 mM EGTA, 20 mM Tris, pH 7.0.

Isotonic relaxing solutions consisted of 1:1 mixtures of the above solutions with 6.75% $MgCl_2$ (i.e. SW/$MgCl_2$, etc.).

Hypotonic solutions typically consisted of 35% or 40% dilutions of isotonic or isotonic relaxing solutions with distilled water (i.e. 40% SW/$MgCl_2$, 0.35 M sorbitol, etc.).

For tests using hypotonic CaF ASW/$MgCl_2$ or 0.35 M sorbitol solutions, veligers were first washed 4-5 times in isotonic CaF ASW or 1 M sorbitol solutions. CaF ASW-washed veligers were treated with CaF ASW/$MgCl_2$ before perfusion of hypotonic solution.

Carboxyfluorescein diacetate and/or carbocyanine labelling of paddle cilia

$DiIC_{16}$ stock (1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Inc., Eugene, OR) consisted of a saturated solution (8 mg/ml) in ethanol. The suspension was vortexed and the supernatant was diluted 1:100 in 1 M sorbitol to give a final DiI concentration of about 50 μM. CFDA stock (5-(and-6)-carboxyfluorescein diacetate, mixed isomers; Molecular Probes, Inc.) was 1 mg/ml in DMSO. Stock was diluted 1:100 in 1 M sorbitol to give a final CFDA concentration of 10 μg/ml.

Veligers in SW were treated with DiI/sorbitol for 20 min, then CFDA/sorbitol for 5 min. Alternatively, veligers were treated in DiI/sorbitol alone for 20 min. After loading, veligers were washed in SW and allowed to swim until used. Paddle cilia were induced by standard 35% dilutions of isotonic solutions.

Demembranation of paddle cilia

Triton X-100 (Pierce, Rockford, IL), Brij-58 (Polyoxyethylene 20 cetyl ether) (Sigma, St. Louis, MO), saponin (Sigma), Nonidet P40 (Particle Data Labs, Elmhurst, IL), and octyl glucopyranoside (OGP) were used at 0.1-2% in the same hypotonic solutions used to induce paddle cilia.

Effects of Ca or Sr on axonemes

Veligers with normal cilia or paddle cilia were treated with 0.1% Triton X-100 in wash solution (WS: 150 mM KCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 30 mM PIPES, pH 6.9) to remove ciliary membranes. Veligers were washed in WS, then perfused with either 0.1 M Ca or Sr in WS (pH 6.9) for at least 5 min. The shape of the axonemes was observed by phase-contrast or darkfield microscopy.

Light microscopy

Perfusion slides of veligers were examined with Zeiss microscopes using phase-contrast, darkfield or fluorescence optics with 40/0.75 or 63/1.4 objectives. Photographs were taken with an Olympus OM 2N camera on Kodak Tech Pan 35 mm film (2415) using an Olympus T32 electronic flash inserted in the microscope light path. Video recording was done with a DAGE 67 M Newvicon camera (DAGE-MTI, Michigan City, IN) which could be synchronized to a strobe flash (Chadwick-Helmuth Strobex 8440, El Monte, CA). Fluorescence was imaged with a DAGE 65 SIT video camera. The effects of detergents on axonemal shape and ciliary membranes of CFDA and/or DiI-labelled paddle cilia were followed by switching back and forth between phase-contrast (or darkfield) and fluorescence optics. Video images were recorded with a GYYR (model 2051; Odetics, Anaheim, CA) or SONY (model 5800 H) VCR using a frame/field counter (QSI model VFF 6030, Woburn, MA). Video fields or frames were either photographed from a monitor or averaged and processed using Image-1 (Universal Imaging Corp., West Chester, PA) with a SONY video printer.

Electron microscopy

Crepidula veligers were relaxed in isotonic SW/$MgCl_2$. Controls were fixed in 2.5% glutaraldehyde, 1% paraformaldehyde, 1% osmium tetroxide, 75 mM NaCl, 10 mM $CaCl_2$, 0.2 M sodium cacodylate (pH 7.7) for 1 h at 0°C. A second group of larvae was treated with 35% SW/$MgCl_2$ for 5 min to induce paddle cilia, then fixed as above. A third group was treated with 35% SW/$MgCl_2$, followed by 0.1% Triton X-100 in 35% SW/$MgCl_2$ for 4 min to disrupt paddle ciliary membranes, then fixed as above. All batches were washed in 0.3 M NaCl, 0.2 M sodium cacodylate (pH 7.7) for 1 h at 0°C, postfixed in 1% osmium tetroxide, 0.38 M NaCl, 0.1 M sodium cacodylate (pH 7.7) for 15 min at 0°C, washed in distilled water for 30 min at 0°C, then left overnight in 1% aqueous uranyl acetate at 4°C. Veligers were dehydrated in ethanol and flat-embedded in Araldite. Single veligers were mounted on stubs and sectioned in known orientations with a diamond knife. Sections were stained with lead and uranyl salts, and viewed at 80 kV with a Zeiss 10CA electron microscope in the Central Microscope Facility of the MBL.

RESULTS

Treatment of *Crepidula* veligers with isotonic relaxing solutions (see Materials and methods) prevents muscular retraction of the velum and allows continuous beating of the velar cilia. The preoral cilia, studied here, consist of a row of closely spaced compound ciliary organelles. Each compound cilium is a blade-shaped group of 20-30 cilia, tapered toward the tip and 60-70 μm long, that beats together as a unit (Fig. 1A). The power (effective) stroke is directed outwards and backwards from the velum, propelling the larva velum-first through the water (Carter, 1926). No paddles or distal expansions of the velar ciliary membranes are present in isotonic solutions with or without $MgCl_2$.

Hypotonic induction of typical paddle cilia

Perfusion of 35% or 40% SW or ASW with or without $MgCl_2$ (see Materials and methods) readily induces formation of paddle cilia. Typically, phase-dense knobs appear first along the length of the compound cilia, which slow down and stop in an upright position at the end of the recovery stroke (Fig. 1). The membrane expansions enlarge

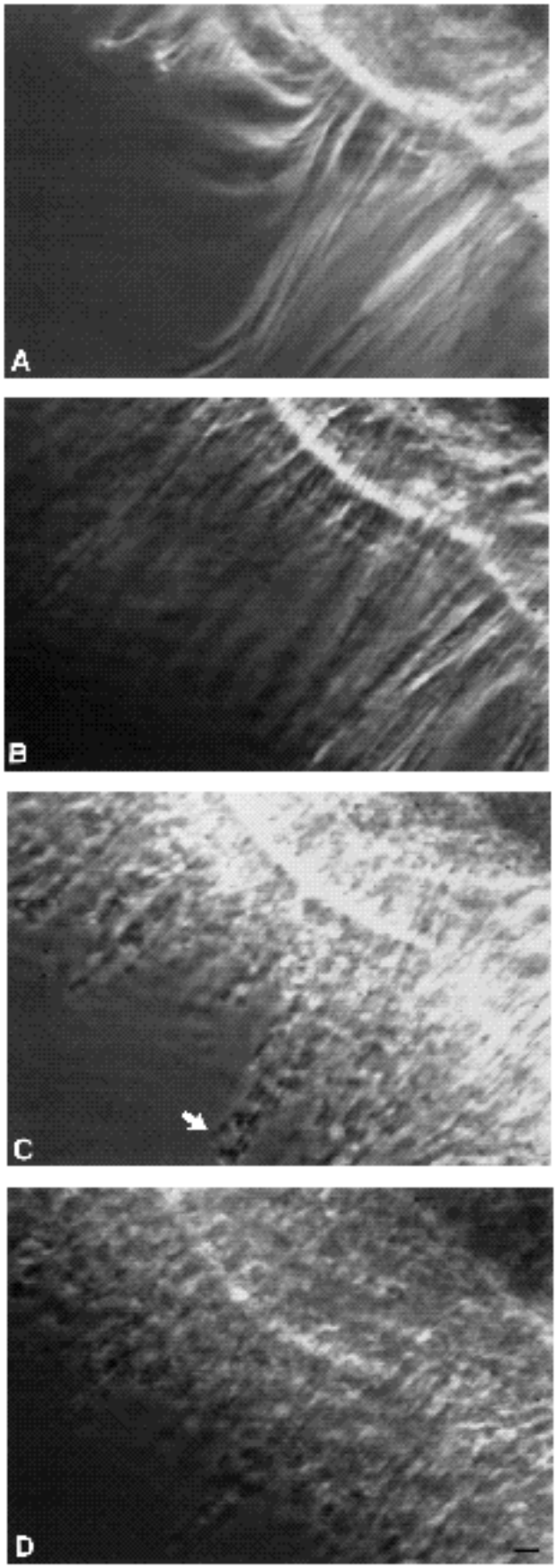

Fig. 1. Hypotonic induction of paddle cilia in the preoral ciliary band of a *Crepidula* veliger. (A) In isotonic ASW/$MgCl_2$ the compound cilia beat vigorously, normal to the page. (B) Perfusion of 40% ASW/$MgCl_2$ causes the cilia to stop in an upright position and become vesiculated. (C) The compound cilia shorten and fray apart into individual cilia with discoid expansions, 2-3 μm in diameter, at their tips (arrow). (D) After 40 s in hypotonic solution that cilia have shortened to about half their length, forming a dense mat of paddles near the velar surface. Phase-contrast optics, video prints; Bar, 4 μm.

slightly and travel towards the ciliary base as the compound cilia shorten and fray apart into single cilia (Fig. 1). Individual cilia, where discernible, are never vesiculated along their length, but bear only a single discoid expansion, 2-3 μm in diameter, at the tip. This paddle travels proximally without changing diameter as the cilium shortens. The initial vesiculation that appears along the length of the compound cilia probably represents terminal paddles formed by component cilia of differing lengths. Within 1-2 min of exposure to hypotonic solution, many cilia have shortened to about half their original length, forming a dense mat of paddles near the velar surface (Fig. 1D).

Under phase-contrast the paddles appear in side view as light grey disks (the distended membrane) with a dark circular rim (the coiled axoneme) (Fig. 2). One edge of the rim continues straight into the ciliary shaft, giving the paddle an asymmetric, cane-shaped profile that reveals the direction of axonemal coiling (Fig. 2; see also Fig. 7). In edge view the paddles are nearly flat or slightly convex (Fig. 2). The flat sides of the paddles lie in the plane of beat, i.e. normal to the row of velar cilia. Phase-contrast microscopy of paddle cilia in *Crepidula* veligers and azide-inhibited *Mulinia* veligers (below) shows that the cane-shaped paddles face toward the veliger's body. Therefore, the axonemes coil preferentially in the direction of the power stroke (see also Electron microscopy below). CFDA-filled or DiI-labelled paddle cilia appear as uniformly fluorescent disks atop bright shafts, illustrating the intraciliary space in the case of CFDA, or the enclosing membrane with DiI (see Fig. 9B).

As reported previously (Short and Tamm, 1991), paddle cilia induced by hypotonic solutions usually regain their normal appearance if larvae are returned to isotonic SW. In such cases, the terminal paddles move distally and eventually disappear at the tips as ciliary length increases. Motility often resumes, but is weaker and abnormal. The reversibility of paddle cilia formation is sometimes evident in SW of intermediate hypotonicity. For example, we observed a shortened paddle cilium in 45% SW that elongated, only losing its paddle when it approached its original length.

Other types of hypotonically induced ciliary shape changes

In addition to typical paddle cilia described above, other modifications of ciliary shape occur as a result of hypotonic solutions. In 40-45% SW the distal end of the axoneme sometimes folds back upon itself within the ciliary membrane like a hairpin, forming a symmetric, teardrop-shaped loop at the tip of the cilium (Fig. 3). The cilium appears

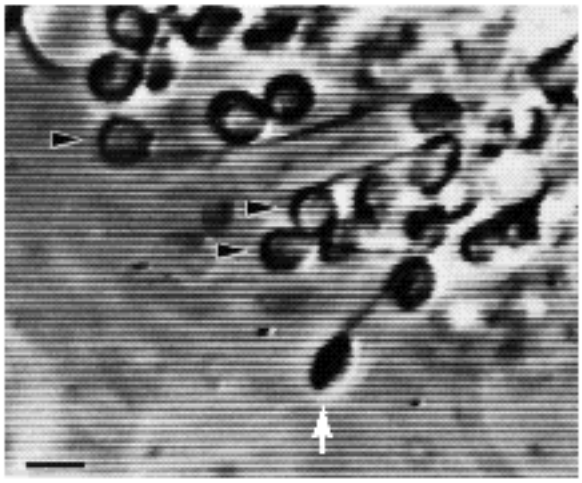

Fig. 2. Phase-contrast image of *Crepidula* paddle cilia induced by 30% SW/$MgCl_2$. The axonemes coil toward one side of the shaft, giving the paddles an asymmetric cane-shaped appearance in profile (arrowheads). Viewed almost edge-on, the paddles appear nearly flat and phase-dense (arrow). The paddles are oriented in different directions due to flattening of the preparation with the coverslip. Bar, 4 μm.

thicker where the distal end of the axoneme overlaps the shaft. The U-shaped bend at the tip of the axoneme may move in either direction. If the bend moves proximally, the cilium shortens and the region of axonemal overlap increases. If the bend travels in the opposite direction, the looped tip moves distally, decreasing the amount of overlap (Fig. 3). When the end of the axoneme reaches the loop, the shape of the tip abruptly changes from a symmetric teardrop into an asymmetric, cane-shaped paddle. Once this occurs, the paddle moves proximally and the cilium shortens due to coiling of the axoneme within the distal membrane (as described above) (Fig. 3). We have never seen the reverse of this transformation, i.e. a typical cane-shaped paddle becoming a hairpin loop.

Very rarely, we have seen a bulbous expansion of the ciliary membrane appear at some location along the length of a cilium. The cilium then bends at this point and folds over upon itself, forming a teardrop-shaped tip. The membrane of the folded-over segment of the cilium appears to fuse with that of the shaft, forming a hairpin-shaped distal region similar to the hairpin loops described above. This process presumably represents the formation of the hairpin-shaped cilia.

Electron microscopy of paddle cilia

In longitudinal thin sections cut parallel to the plane of beat, most paddle cilia stand at the end of the recovery stroke (Fig. 4). The flat sides of the paddles lie in the plane of section. The straight shaft of the axoneme enters the paddle on the recovery stroke side and coils in the direction of the effective stroke (Fig. 4). These results confirm light microscopic observations on the direction of axonemal coiling (see also below).

Transverse sections through paddles, cut parallel to the velar surface, show up to 3 complete coils of the axoneme within the expanded membrane (Figs 5, 6). The axis of the central-pair microtubules is oriented at right angles to the plane of coiling. Doublet microtubules 5 and 6, recognized by the bridge between them, always face the inside of the axonemal coils (Fig. 5). Therefore, the axonemes consistently coil toward the doublet 5-6 side. As in many other cilia, this is the direction of the effective stroke.

Transverse sections also show that the axoneme is intact in the straight shaft but becomes disorganized in the distal coiled region. The axoneme usually splits into several groups of doublets on either side of the central pair, i.e. in the plane of coiling (Figs 5, 6). Adjacent doublets within groups are frequently aligned in almost flat strips. parallel to the direction of coiling. The axoneme usually becomes progressively more disrupted in successive coils within a paddle. The coil with the most intact axoneme is presumably where the shaft first enters the paddle.

We used this progressive splitting of the axoneme from coil to coil to determine the handedness of the axonemal helix in paddles. As viewed in the direction of the effective stroke and from base to tip of the cilium, coiling of the axoneme to the right or to the left of the shaft would represent right-handed or left-handed helices, respectively. We found both directions of axonemal coiling in the same transverse sections through paddle cilia (Fig. 6). Therefore, the axoneme does not seem to have a preference to coil to one side or the other of the beat plane, i.e. towards doublets 2-4 or 7-9.

Separation of doublets in the distal coiled region of axonemes is often evident in longitudinal sections through

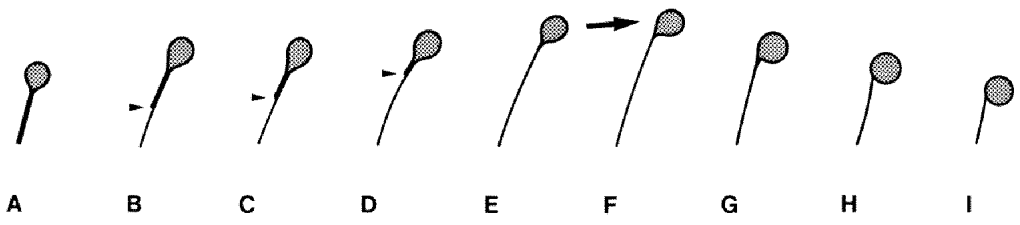

Fig. 3. Video tracings of hairpin loop-to-paddle transformation of the distal region of a single velar cilium (*Crepidula*) in 45% SW/$MgCl_2$. The proximal end of the cilium was obscured by other cilia and is omitted. (A) The axoneme is folded back upon itself (thicker shaft) to form a symmetric hairpin loop. (B-E) The loop travels distally, elongating the cilium and simultaneously decreasing the region of overlap (arrowheads). (E-F) When the end of the axoneme reaches the loop, an asymmetric cane-shaped paddle suddenly appears. (G-I) The paddle moves proximally due to progressive coiling of the end of the axoneme within the membrane expansion. Time from (A): (B) 8 s, (C) 9.3 s, (D) 11 s, (E) 14.3 s, (F) 15.5 s, (G) 16 s, (H) 18 s, (I) 24.8 s.

paddles. These sections also reveal occasional breaks in the doublet microtubules, as well as fragments of microtubules within paddles, indicating that curvature-induced stress exceeds the elasticity of the microtubules.

Possible role of Ca influx or proton efflux in paddle formation

Veligers were treated with hypotonic Ca-free solutions at neutral or low pH to test whether Ca influx or proton efflux plays a role in formation of paddle cilia.

We first established that isotonic solutions of CaF ASW/$MgCl_2$, 1 M sorbitol (pH 3.7), or 1 M sorbitol with 1 mM EGTA, 20 mM Tris (pH 7.0) do not induce paddle cilia or disrupt ciliary beating. Subsequent perfusion of 35% CaF ASW/$MgCl_2$ induces paddle cilia, but is less effective than 35% SW/$MgCl_2$. However, 20% CaF ASW/$MgCl_2$, or 35% dilutions of the above 1 M sorbitol solutions, induces paddle cilia which are similar to those formed in hypotonic SW solutions with Ca at normal pH. These findings indicate that axonemal coiling and formation of paddle cilia

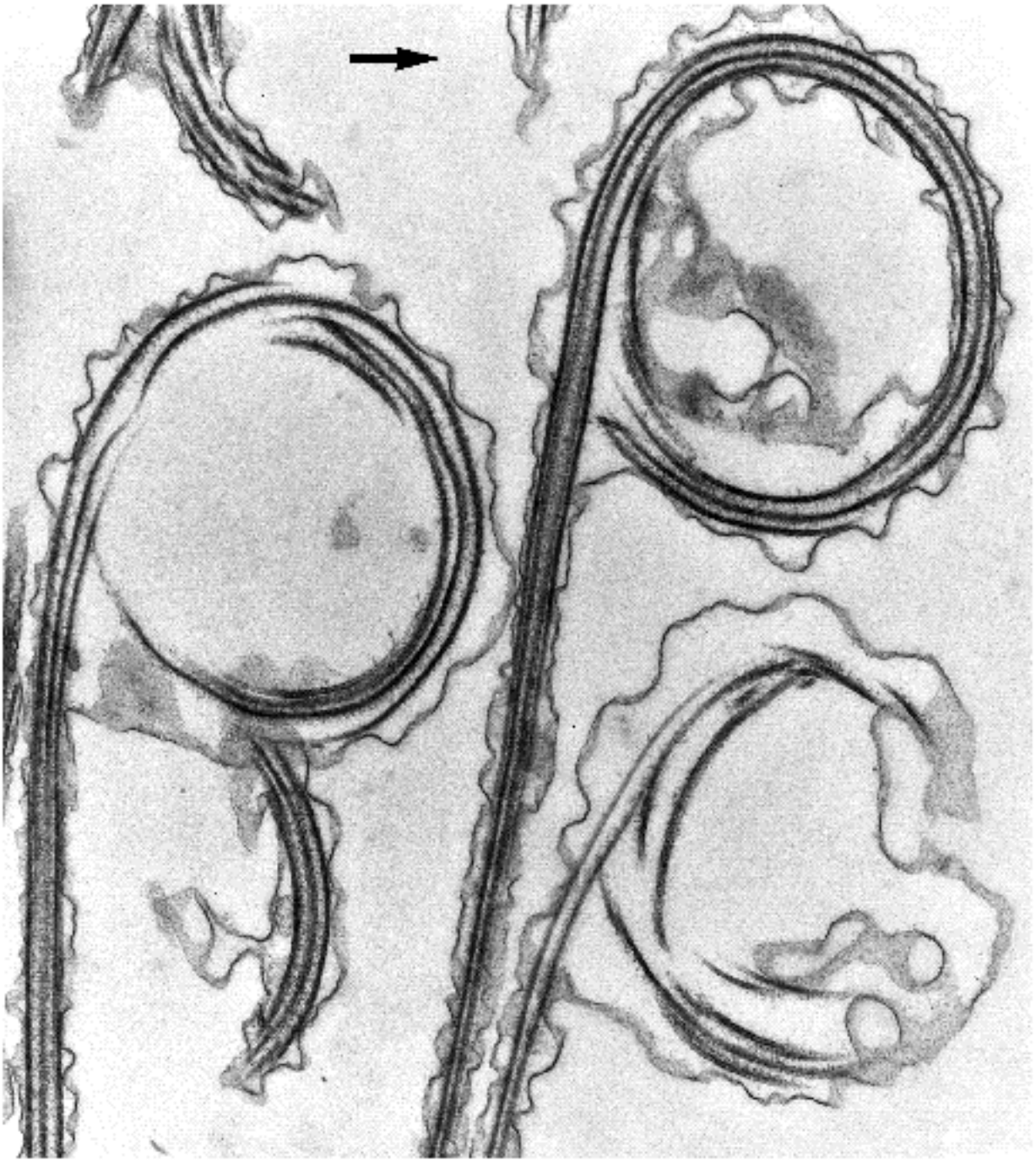

Fig. 4. Electron micrograph of *Crepidula* paddle cilia sectioned parallel to the plane of beat. The distal ends of the axonemes coil to the right in the direction of the effective stroke (arrow), resulting in cane-shaped profiles. ×33,500.

under hypotonic conditions are not dependent on Ca influx or proton efflux, as proposed by Short and Tamm (1991).

In hypotonic CaF ASW/$MgCl_2$ paddle cilia often continue to beat weakly, and do not shorten as much as typical paddle cilia formed in hypotonic SW. Although hypotonic CaF ASW/$MgCl_2$ initially induces paddles of normal size, large swellings of the cell membrane often develop at the base of each compound cilium. These swellings slowly move up the cilia, enclosing many of the axonemes inside a common, fused membrane. As a result, a single large expansion of the membrane encloses the distal ends of these axonemes, which curve (but do not coil) under the ballooned membrane. Hypotonic sorbitol solutions (0.35 M, pH 2.6, or 0.35 M with 0.35 mM EGTA and 7 mM Tris, pH 7.0) did not result in balloon-like expansions of fused ciliary membranes. However, such membrane swellings enclosing many axonemes could be induced by hypotonic SW after prolonged exposure to isotonic 1 M sorbitol solutions. Paddle cilia induced in hypotonic sorbitol solutions, like those in hypotonic CaF ASW, often continue to beat weakly.

Direction of axonemal coiling vs active beating

Two- to three-day-old *Mulinia* veligers were used to test whether the uniform direction of axonemal coiling in paddle cilia (i.e. towards the effective stroke; see above) is dependent on active cyclic beating prior to induction of paddle cilia by hypotonic solutions. As in previous experiments with 2-day-old *Spisula* veligers (Short and Tamm, 1991), extremely hypotonic solutions (i.e. 20% SW) are required to induce paddle cilia in these young larvae. We inhibited beating by two methods prior to hypotonic treatment. First,

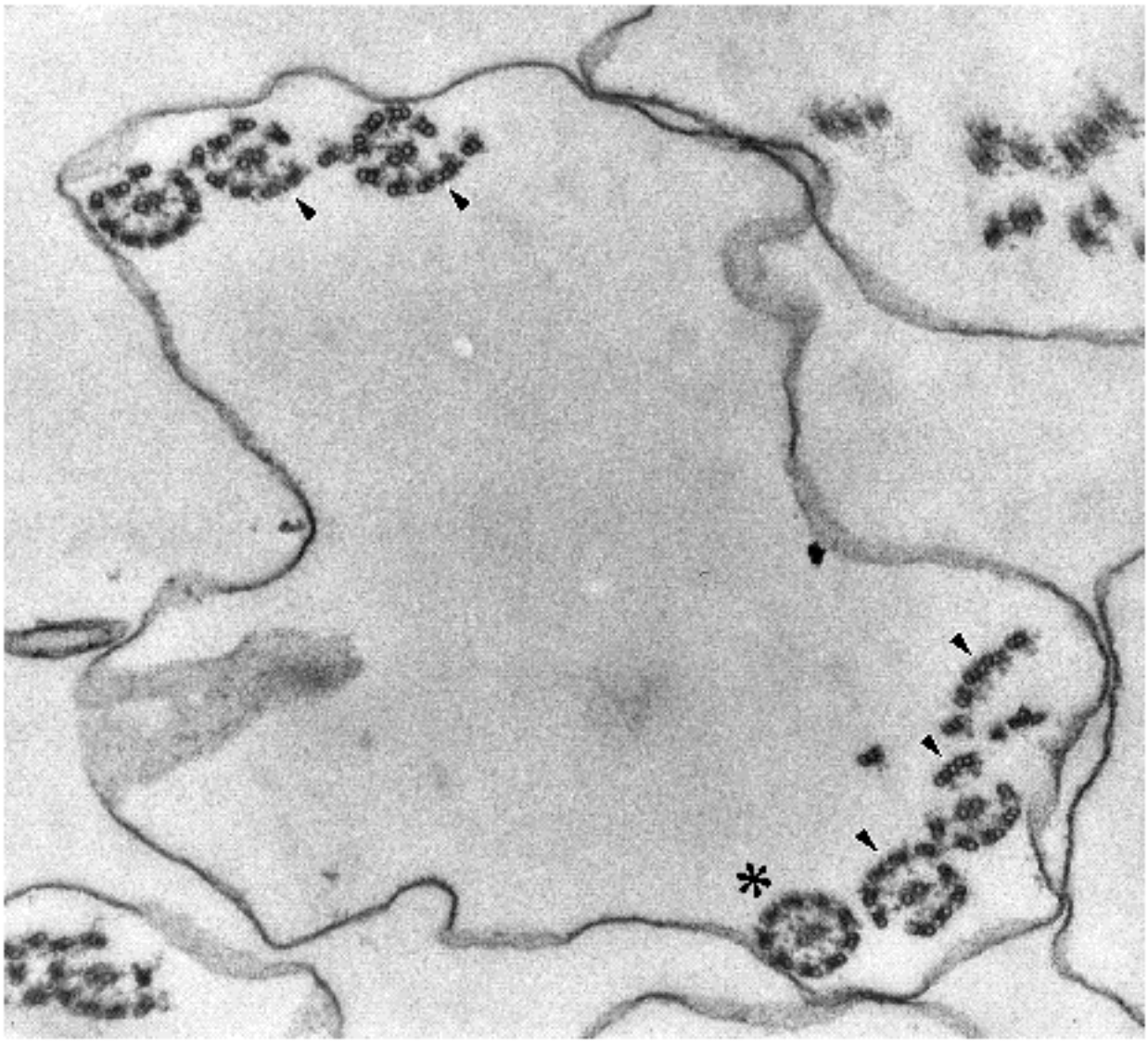

Fig. 5. Transverse thin section through a coiled axoneme in a paddle (*Crepidula*). The section is viewed from base to tip of the cilium with the effective stroke direction toward the upper left. The axoneme is intact where it enters the paddle (asterisk) but becomes progressively more disorganized in successive coils, splitting into groups of doublets on either side of the central pair (oriented normal to the plane of coiling). Doublet microtubules 5-5 face the inside of the coils (arrowheads, 5-6 bridge), showing that the axoneme coils toward this side. ×69,100.

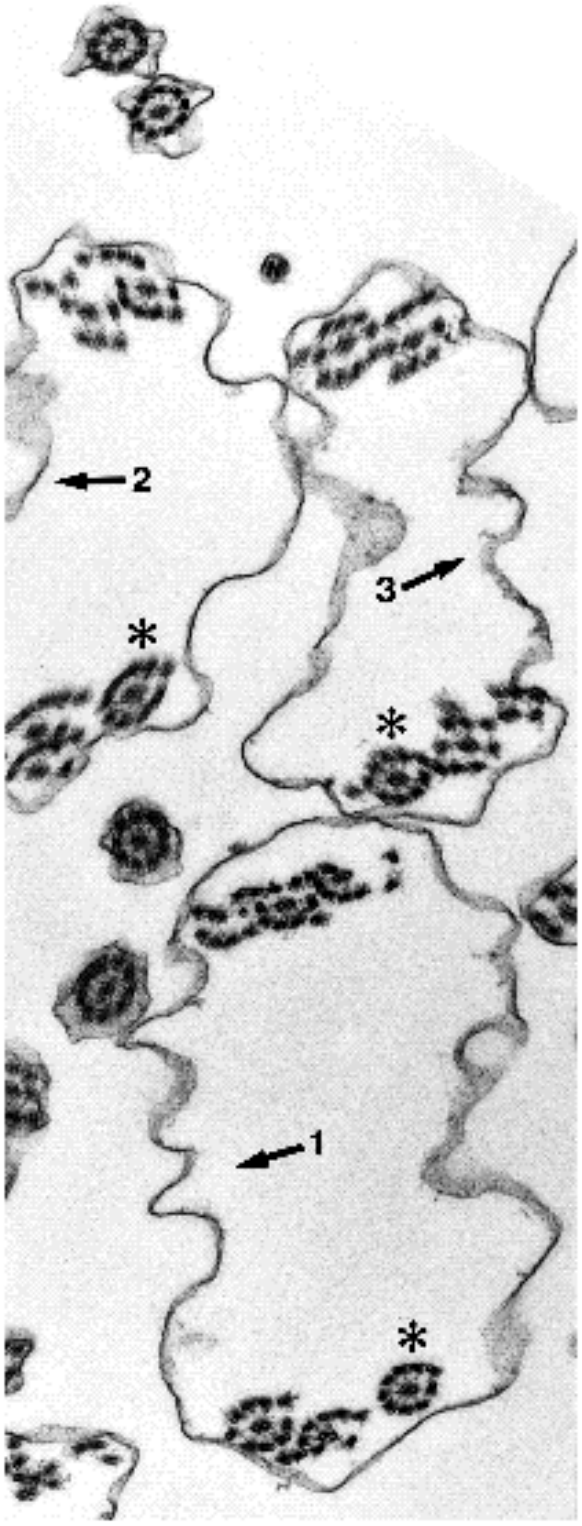

Fig. 6. Transverse thin section through adjacent paddle cilia with axonemes coiling in the same direction (towards top) but with opposite handedness (*Crepidula*). The section is viewed from base to tip of the cilia with the effective stroke directed toward the top. The axonemes are intact where they enter the paddles (asterisks), but coil to the left in paddles 1 and 2 (left-handed helix), and to the right in paddle 3 (right-handed helix) (see arrows). ×36,400.

swimming veligers were lightly fixed in 0.012% glutaraldehyde in ASW for 5 min. All ciliary motility stopped immediately. Phase-contrast observations of fixed larvae washed in isotonic ASW showed that most velar cilia were normal in shape and length. Treatment of fixed larvae with distilled water, however, induced formation of typical, shortened paddle cilia. In a second experiment, swimming veligers were treated with 100 mM sodium azide in ASW. After 10-20 min, no motility was evident. Phase-contrast examination showed normal but immotile velar cilia. Subsequent treatment with 50 mM sodium azide in distilled water induced formation of typical paddle cilia without allowing recovery of motility.

In both glutaraldehyde-fixed (not shown) and azide-treated veligers (Fig. 7), the cane-shaped paddles face uniformly backwards toward the larval body. The axonemes therefore coil uniformly in the direction of the effective stroke, as described above for cilia that were motile before hypotonic treatment. These findings show that the direction of axonemal coiling is not dependent upon cyclic beating or active microtubule sliding prior to induction of paddles.

Effects of membrane disruption on the shape of paddle cilia

The membranes of paddle cilia were disrupted with detergents to test whether axonemal coiling is dependent on membrane integrity. Perfusion of pre-formed paddles with 0.1% Triton-X 100, 0.1% Nonidet P40, or 25-50 mM OGP in any of the hypotonic solutions used to induce paddle cilia (see above) resulted in loss of paddles. Paddles were lost first in longer cilia that projected into the flow of detergent, and later in shorter, more densely packed paddle cilia near the velar surface. Typically, paddles moved distally and disappeared at the tip as the axonemes lengthened like unwind-

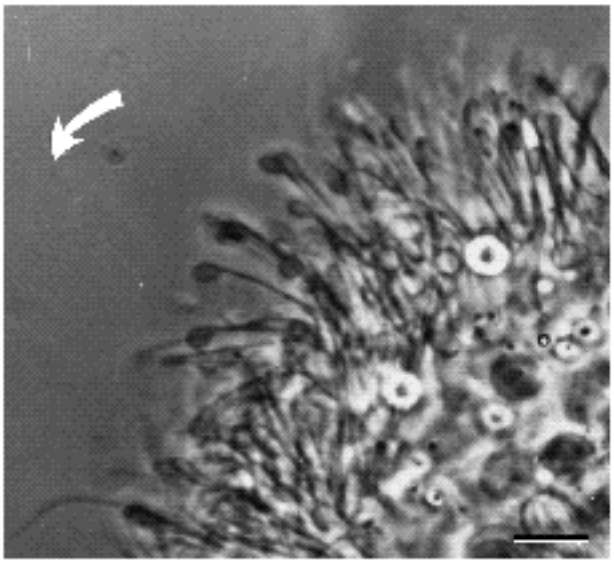

Fig. 7. Hypotonically induced paddle cilia in an azide-inhibited, immotile veliger of *Mulinia.* Lateral view of part of the velum shows cane-shaped paddles facing downward toward the body (out of view) in the effective stroke direction (arrows). Bar, 5.4 μm.

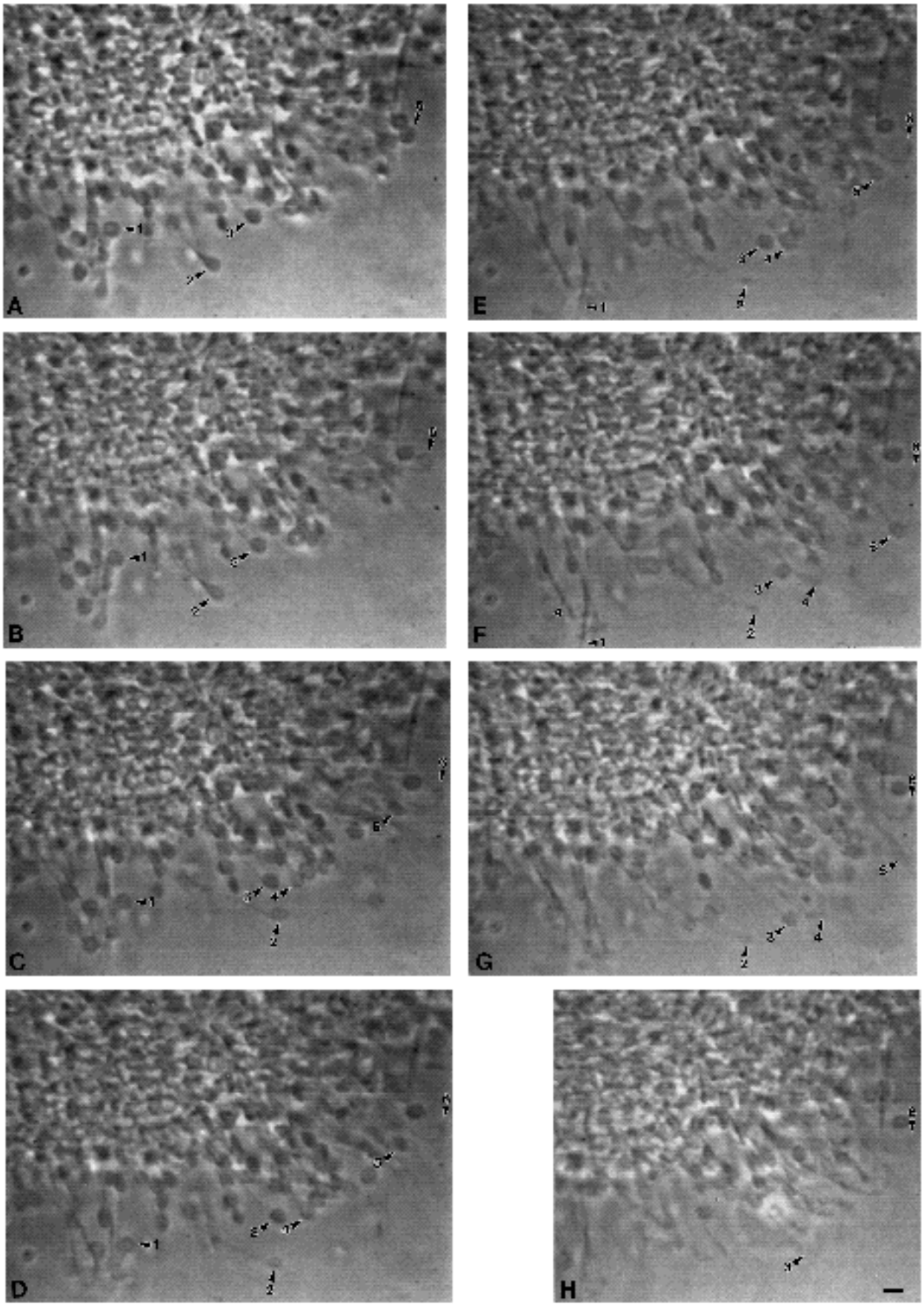

Fig. 8

Fig. 8. Detergent-induced uncoiling of paddle ciliary axonemes in *Crepidula*. (A) Paddle cilia induced by 40% SW/$MgCl_2$. Numbered paddles are followed in successive prints. (B) Perfusion of 0.1% Triton X-100 in 40% SW/$MgCl_2$ initially causes paddles to move distally, elongating the cilia (nos 1, A-D; 2, A-C; 3, A-G; 4, C-E; 5, C-F). The paddles disappear at the tip as the distal end of the shaft uncoils (1, E; 2, D; 3, H; 4, F; 5, G; 6, B). Time from (A): (B) 1 s, (C) 4 s, (D) 5 s, (E) 6 s, (F) 6.5 s, (G) 7 s, (H) 9 s. Phase-contrast optics, video prints; Bar, 4 μm.

ing springs (Fig. 8). Sometimes, however, detergent perfusion caused the paddle to "pop" suddenly, releasing the coiled axoneme, which sprang out in a wide curved arc. Uncoiling and straightening of the distal region of the axonemes resulted in an increase in length to approximately that of the original cilia before hypotonic treatment. In one clear example, a paddle uncoiled in detergent solution to a length of 3 times the original circumference of the paddle, consistent with electron micrographs showing 3 coils of the axoneme within paddles (see above). The straightened or slightly curved distal region of uncoiled axonemes often appeared finer than the proximal shaft, probably due to its splitting during coiling. Paddle cilia induced by hypotonic CaF ASW/$MgCl_2$, but not by other solutions, typically underwent a brief burst of beating just after detergent treatment as the paddles were lost. Beating then slowed and soon stopped.

The effects of detergents on ciliary membranes were visualized using paddle cilia pre-labelled with the fluorescent probes DiI and CFDA (Fig. 9). Alternate observations by phase-contrast and fluorescence microscopy showed that detergent first caused complete loss of CFDA fluorescence, indicating permeabilization of the ciliary membrane. This was followed by loss of DiI staining from the distal region of the cilia, indicating removal of the paddle membranes (Fig. 9D). Uncoiling and lengthening of the axonemes, as described above, occurred at about the same time. DiI fluorescence often persisted for some time in the closely packed proximal region of the cilia (Fig. 9D), but eventually disappeared there as well. By phase-contrast

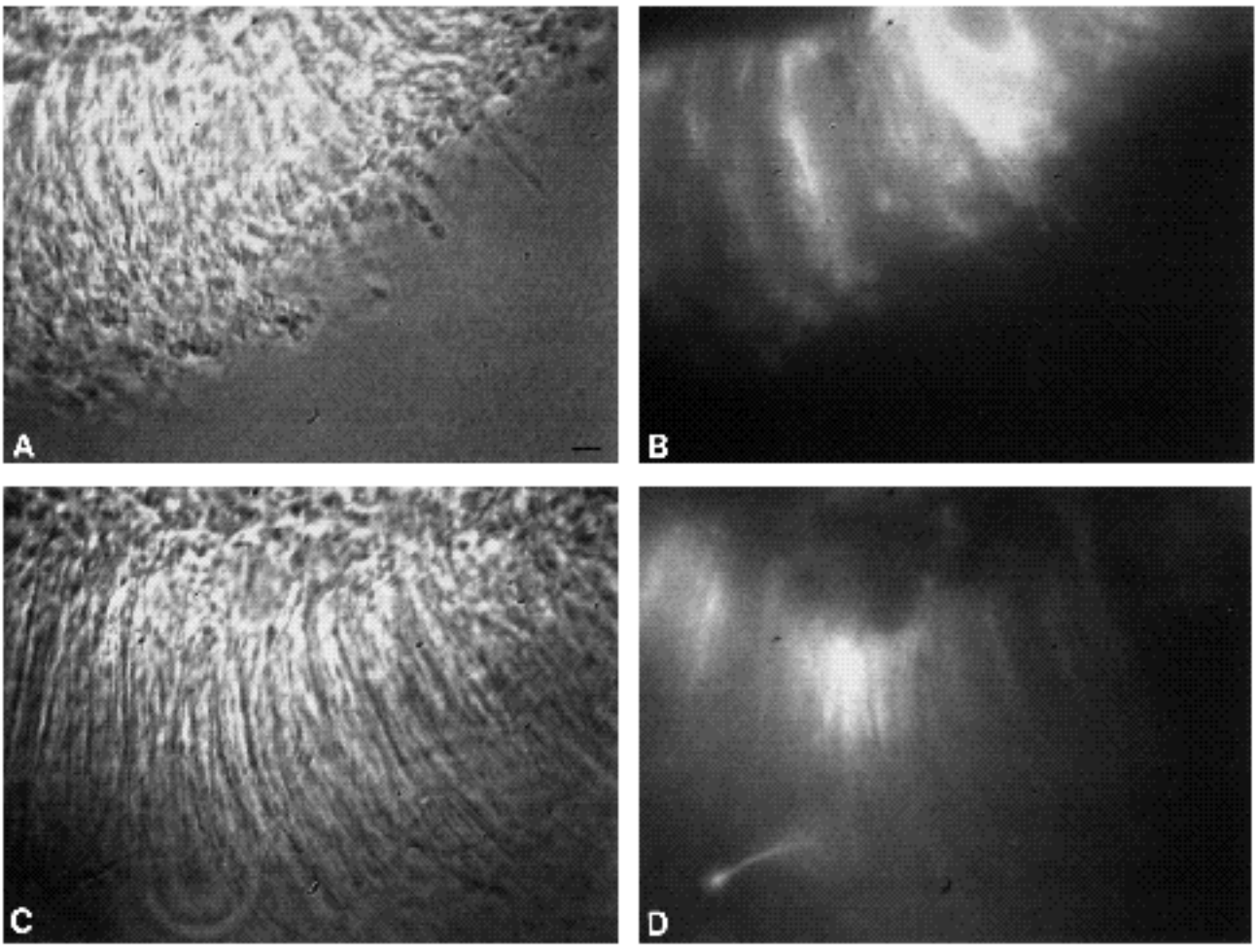

Fig. 9. Effect of detergent on DiI-labelled membranes of paddle cilia induced by 35% SW/$MgCl_2$ (*Crepidula*). (A,B) Before detergent. Phase-contrast (A) and fluorescence (B) of same field, showing fluorescent ciliary shafts and terminal paddles. (C,D) After perfusion of 0.1% Nonidet P40. Phase-contrast (C) shows uncoiled, straightened shafts with fine distal regions. Fluorescence (D) of the same field shows loss of DiI label in the distal regions of the cilia, indicating removal of membrane by detergent. Fluorescence is retained in the more closely packed proximal region of the cilia, but eventually disappears there as well. Note brightly fluorescent detached paddle cilium. Bar, 5 μm.

microscopy, loss of paddles was accompanied by a flow of small particles and debris from the cilia, representing membrane fragments and/or vesicles released by the detergent. Electron microscopy of thin sections through Triton-X-treated paddle cilia showed many bare axonemes without membranes as well as some ciliary cross-sections with membranes.

In some cases a few paddle cilia remained intact after detergent treatment. Fluorescence microscopy showed that these cilia had lost CFDA label but completely retained DiI staining in the paddle and shaft. Therefore, removal of the ciliary membrane, not just permeabilization. is required for uncoiling of axonemes within paddles.

Saponin and Brij-58 (up to 2%) were ineffective in causing uncoiling of axonemes and loss of paddle cilia. Although CFDA fluorescence was fully lost, DiI staining was partially retained, indicating incomplete removal of ciliary membranes by these detergents.

Controls for possible photobleaching of fluorescent probes showed that 10-15 min of continuous illumination with the mercury lamp was required before fluorescence was lost. This exposure time greatly exceeded that used in our experiments.

Effects of Ca and Sr on the shape of demembranated axonemes

We tested whether Ca or Sr can induce coiling of the tips of demembranated velar cilia. Veligers in isotonic SW/$MgCl_2$ were perfused with 0.1% Triton-X 100 in WS. Beating stopped and the flow of particulate debris from the straight, immotile cilia indicated loss of ciliary membranes. Perfusion of WS to remove detergent was followed by perfusion of either 0.1 M Ca or 0.1 M Sr in WS. Despite this high concentration of Ca or Sr, the tips of the demembranated axonemes remained straight and did not coil.

In other experiments, we treated veligers initially with WS (which is hypertonic to SW) to induce paddle cilia. Perfusion of 0.1% Triton-X 100 in WS then led to loss of paddles and uncoiling of axonemes, confirming that detergent successfully removed ciliary membranes. Subsequent treatment with 0.1 M Ca or Sr in WS failed to elicit recoiling or other changes in shape of the straight distal regions of the axonemes. High concentrations of Ca or Sr are therefore ineffective in causing detectable conformational changes of these axonemes.

DISCUSSION

Although paddle cilia or discocilia have been widely reported in a variety of marine invertebrates, and implicated in diverse functions, these ciliary modifications are now recognized to be artifacts of osmotic stress or other non-physiological conditions (Bone et al., 1982; Ehlers and Ehlers, 1978; Nielsen, 1987; Short and Tamm, 1991).

Short and Tamm (1991) proposed that coiling of the distal end of the axoneme under hypotonic conditions is due to a helical conformational change of the ciliary doublet microtubules, triggered by Ca or proton flux through the osmotically stressed ciliary membrane. This model was based on evidence that physiological changes in Ca concentration or pH can induce reversible changes in helical parameters of isolated doublet microtubules in solution (Miki-Noumura and Kamiya, 1976, 1979; Takahashi and Miki-Noumura, 1982).

We show here that axonemal coiling in paddles is not the result of stable conformational changes in doublet microtubules, but is caused by membrane tension acting on elastic axonemes. Removal of the paddle ciliary membranes with Triton X-100 or Nonidet P40, as verified by loss of DiI labelling, results in immediate uncoiling and straightening of the axonemes to approximately their original length before hypotonic treatment. This dramatic unwinding of the coiled axonemes upon disruption of the paddle membrane is most easily explained by a release of elastic strain energy stored in the coiled axoneme.

It is unlikely that detergent-induced uncoiling of the axonemes is due to ionic fluxes which reverse possible conformational changes of the doublet microtubules. Combined CFDA and DiI labelling showed that membrane permeabilization alone is not sufficient to cause uncoiling; instead, complete removal of the distal ciliary membranes is necessary to straighten the axonemes.

Further evidence for the important role of the ciliary membrane in influencing the shape of the axoneme comes from our observations of other types of hypotonically induced ciliary changes. The hairpin loop at the tip of cilia in 40-45% SW represents a planar, U-shaped bend near the distal end of the axoneme. No helical coiling is evident. The bend can travel in either direction. If it moves proximally, the cilium shortens; if it travels distally, the cilium elongates. Movements of a planar bend along the axoneme are most easily accounted for by changes in membrane tension.

The abrupt transformation of a distally traveling hairpin bend into a cane-shaped paddle, followed by helical coiling of the axoneme and shortening of the cilium, demonstrates different types of conformational changes under apparently identical ionic conditions. These observations can be accounted for by membrane tension if the folded-over part of the looped axoneme is held parallel to the shaft by the enclosing membrane sleeve. When the hairpin bend nears the distal end of the axoneme, eliminating the overlap, the curved tip would be free to coil under imposed membrane tension, forming typical paddles. As expected, the reverse transformation from paddle cilia to hairpin-shaped folds was never observed. These changes in axonemal shape do not require fluctuations in membrane tension, but could occur under constant tension if hairpin loops and helical coils exert different elastic forces on the membrane. Hairpin loop cilia presumably originate by folding back of a cilium upon itself at a membrane varicosity, followed by fusion of the adjoining membranes (as observed). Similar hairpin-shaped folds of axonemes surrounded by a common fused membrane have been produced in flatworm spermatozoa by hypotonic media (Justine and Mattei, 1988).

Our finding that typical paddle cilia are induced by hypotonic Ca-free solutions at normal or low pH shows that axonemal coiling is not dependent on Ca influx or proton efflux as proposed by Short and Tamm (1991). Conversely, demembranated axonemes do not coil in the presence of high Ca concentrations, further discrediting a Ca-induced

conformational change of doublet microtubules as the cause of paddle formation.

The influence of the enclosing membrane on the shape of the axoneme is further demonstrated by the ballooned, fused ciliary membranes that develop in Ca-free ASW. The distal ends of the axonemes follow the curved surface of the bulbous membrane expansion, and are not tightly coiled as in single paddle cilia. Axonemal shape thus seems to depend only on the geometry and tensile properties of the enclosing membrane. Helical coiling of the axoneme is therefore imposed by mechanical stress from the outside and is not the result of internal structural rearrangements of the doublet microtubules.

Membrane tension model for axonemal coiling

We observed that the paddle is not spherical, but nearly flat or disk-shaped, indicating that it is not the result of osmotic swelling of the distal ciliary membrane. The constant diameter of the paddle as it moves proximally also indicates that the paddle is not a hypo-osmotic swelling phenomenon. Instead, the source of membrane tension at the ciliary tip is most probably the hypo-osmotic swelling of the body of the ciliated cell. This forced increase in cell surface area should increase plasma membrane tension generally and cause withdrawal of membrane into the soma from thin cellular projections such as cilia.

It is likely that hypotonic solutions first induce osmotic swelling of the ciliated cell body, which in turn pulls ciliary membranes towards the cell surface. The tip of the axoneme in most cilia is narrower and less well-organized that the shaft because only singlet microtubules (A-tubules) are present, and nexin links, radial spokes, and 9-fold symmetry are lacking. In addition, the bridges between axonemal microtubules and the ciliary membrane are weak or missing at the tip (Dentler, 1981; Child, 1961; Pitelka and Child, 1964). Increased tension of the ciliary membrane on the axoneme should therefore first cause bending or coiling of the tip of the axoneme within a distal expansion of the ciliary membrane. Coiling of the axoneme should proceed further until an equilibrium is reached where the elastic restoring force of the accumulated coils balances the tensile stress of the surrounding membrane.

This model requires that the ciliary membrane be able to transmit tensile force from the base to the tip of the axoneme. Yet no obvious cytoskeleton is visible on the cytoplasmic side of ciliary membranes. Biochemical and electron microscopic studies on molluscan gill ciliary membranes, however, have demonstrated the presence of a tubulin-containing lipid-protein structural complex that constitutes an integral protein skeleton in the ciliary membrane (Stephens, 1985, 1986; Stephens et al., 1987). This membrane skeleton might provide sufficient rigidity for transmission of mechanical stimuli to the basal region or somatic membrane in mechanosensitive cilia (Stephens et al., 1987). Furthermore, a recent report indicates the possibility of a membrane-microtubule motor present in cilia and flagella (Kozminski et al., unpublished data). An integral membrane skeleton in velar cilia could provide the necessary force to coil the distal end of the axoneme during hypotonically induced retraction of the ciliary membrane into the plasma membrane.

Axonemal coiling and stiffness

Our finding that axonemes in paddles coil uniformly in the direction of the effective stroke (i.e. towards doublets 5-6), even when beating is inhibited, suggests that axonemes possess an intrinsic asymmetry in stiffness (flexural rigidity) in the beat plane, such that stiffness for passive bending in the recovery stroke direction is greater than that for bending in the effective stroke direction. Because this presumed difference in stiffness for bending in opposite directions is independent of active microtubule sliding, it may reside in the permanent structural organization of the axoneme. The nature of such axonemal component(s) is unknown.

A greater stiffness of the cilium for bending in the recovery stroke direction makes sense with regard to the asymmetrical beat cycle of cilia. Cilia move rapidly through the effective stroke as more or less rigid rods, but are drawn back by a distally propagating bend during the recovery stroke as though they were limp or readily flexible (Sleigh, 1974). Increased stiffness of the axoneme for bending away from the effective stroke direction should provide greater rigidity to resist viscous drag of the medium that pushes the distal part of the cilium backwards during the effective stroke.

However, experimental measurement of the flexural rigidity of the compound abfrontal gill cilium of *Mytilus* using a glass microneedle showed no such directional asymmetry: the stiffness was similar irrespective of the direction of imposed bending, the phase of the beat cycle, or the region of the cilium to which the needle was applied (Baba, 1972). This finding is difficult to reconcile with the form of the ciliary beat cycle, and with our interpretation of the pattern of axonemal coiling in paddle cilia. It should be noted, however, that the beat pattern of the *Mytilus* abfrontal cilium is atypical: “the unrolling recovery stroke occupies a considerably smaller part of the whole cycle than the extended ‘effective’ stroke; in fact, the cilium may move exceedingly slowly or even stop during the effective stroke, so that the usual terminology for the two phases of the cycle does not seem very appropriate” (Sleigh, 1974, p. 86). Measurements of the flexural rigidity of this unusual cilium may not be applicable to cilia with a typical beat cycle. In contrast, Okuno and Hiramoto (1979) found a 3- to 4-fold variation in stiffness around the axis of live echinoderm sperm flagella, but these measurements were not related to the wave plane or ultrastructural organization.

A more serious inconsistency with a possible intrinsic difference in axonemal stiffness related to the asymmetry of the beat cycle comes from our earlier work on ctenophore comb plate cilia. We found that 180° reversal in beat direction of comb plates is not accompanied by rotation of the axonemes (Tamm and Tamm, 1981). A polarized effective-recovery stroke beat cycle can thus occur in opposite directions with respect to a fixed axonemal structure. Clearly, if a built-in asymmetry in axonemal stiffness exists in comb plate cilia, it is not functionally related to the beat cycle.

In conclusion, we have taken advantage of paddle cilia to illustrate several important features of the mechanical properties of ciliary axonemes and their enclosing membranes. These results should aid our eventual understanding of the mechanism and control of ciliary function. Paddle

cilia are therefore useful to cell biologists, even though these artifacts are not used by the animals.

We thank Dr Ray Stephens, Boston University Medical School, for the loan of the SIT camera and much helpful advice. We are grateful to Dr Rachel Fink, Mt. Holyoke College, for suggesting the use of DiI to label ciliary membranes. Dr Shinya Inoué, MBL, generously helped us to use his video processing equipment to make prints from video tapes. The work was supported by NIH grant GM-45557.

REFERENCES

Baba, S. A. (1972). Flexural rigidity and elastic constants of cilia. *J. Exp. Biol.* **56,** 459-467.

Bone, Q., Ryan, K. P. and Pulsford, A. (1982). The nature of complex discocilia in the endostyle of *Ciona* (Tunicata: Ascidiacea). *Mikroskopie* (Wein) **39,** 149-153.

Campos, B. and Mann, R. (1988). Discocilia and paddle cilia in the larvae of *Mulinia lateralis* and *Spisula solidissima* (Mollusca: Bivalvia). *Biol. Bull. Mar. Biol. Lab., Woods Hole* **175,** 343-348.

Carter, G. S. (1926). On the nervous control of the velar cilia of the nudibranch veliger. *J. Exp. Biol.* **4,** 1-26.

Child, F. M. (1961). Some aspects of the chemistry of cilia and flagella. *Exp. Cell. Res. Suppl.8*, 47-53.

Conklin, E. G. (1897). The embryology of *Crepidula. J. Morphol.* **134,** 1-226.

Deiner, M. and Tamm, S. L. (1991). Mechanism of paddle cilia formation in molluscan veligers. *Biol. Bull. Mar. Biol. Lab., Woods Hole* **181,** 335-336.

Deiner, M., Tamm, S. L. and Tamm, S. (1992). Structure and mechanism of formation of paddle cilia in molluscan veligers. *5th Int. Congr. Cell Biol., Madrid, Spain,* p. 59.

Dentler, W. L. (1981). Microtubule-membrane interactions in cilia and flagella. *Int. Rev. Cytol.* **72,** 1-47.

Ehlers, U. and Ehlers, B. (1978). Paddle cilia and discociia - genuine structures? *Cell Tiss. Res.* **192,** 489-501.

Justine, J.-L. and Mattei, X. (1988). Bending of 9 + "1" axonemes of flatworm spermatozoa in hypotonic media: an experimental study. *J. Ultrastruct. Mol. Struct. Res.* **100,** 31-38.

Miki-Noumura, T. and Kamiya, R. (1976). Shape of microtubules in solutions. *Exp. Cell Res.* **97,** 451-453.

Miki-Noumura, T. and Kamiya, R. (1979). Conformational change in the outer doublet microtubules from sea urchin sperm flagella. *J. Cell Biol.* **81,** 355-360.

Okuno, M. and Hiramoto, R. (1979). Direct measurements of the stiffness of echinoderm sperm flagella. *J. Exp. Biol.* **79,** 235-243.

Nielsen, C. (1987). Structure and function of metazoan ciliary bands and their phylogenetic significance. *Acta Zool. (Stockh.)* **68,** 205-262.

Pitelka, D. R. and Child, F. M. (1964). The locomotor apparatus of ciliates and flagellates: relations between structure and function. In *Biochemistry and Physiology of Protozoa* (ed. S. H. Hutner), vol. III, pp. 131-198. Academic Press, New York.

Short, G. and Tamm, S. L. (1991). On the nature of paddle cilia and discocilia. *Biol. Bull. Mar. Biol. Lab., Woods Hole* **180,** 466-474.

Sleigh, M. A. (1974). Patterns of movement of cilia and flagella. In *Cilia and Flagella* (ed. M. A. Sleigh), pp. 79-92. New York: Academic Press.

Stephens, R. E. (1985). Evidence for a tubulin-containing lipid-protein structural complex in ciliary membranes. *J. Cell Biol.* **100,** 1082-1090.

Stephens, R. E. (1986). Membrane tubulin. *Biol. Cell* **57,** 95-110.

Stephens, R. E., Oleszko-Szuts, S. and Good, M. J. (1987). Evidence that tubulin forms an integral membrane skeleton in molluscan gill cilia. *J. Cell Sci.* **88,** 527-535.

Takasaki, Y. and Miki-Noumura, T. (1982). Shape of the ciliary doublet microtubule in solution. *J. Mol. Biol.* **158,** 317-324.

Tamm, S. L. and Tamm, S. (1981). Ciliary reversal without rotation of axonemal structures in ctenophore comb plates. *J. Cell Biol.* **89,** 495-509.

(Received 21 September 1992 - Accepted, in revised form, 28 December 1992)