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Effect of Vanadate on Gill Cilia: Switching Mechanism in Ciliary Beat

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Lateral (L) cilia of freshwater mussel (Margaritana margaritifera and Elliptio complanatus) gills can be arrested in one of two unique positions. When treated with 12.5 mM $CaCl_2$ and 10^{-5} M A23187 they arrest in a "hands up" position, ie, pointing frontally. When treated with approximately 10 mM vanadate (V) they arrest in a "hands down" position, ie, pointing abfrontally. L-cilia treated with 12.5 mM $CaCl_2$ and 1 mM NaN_3 also arrest in a "hands down" position; substitution of 20 mM KCl and 1 mM NaN_3 causes cilia to move rapidly and simultaneously to a "hands up" position.

The observations suggest that there are two switching mechanisms for activation of active sliding in ciliary beat one at the end of the recovery stroke and the other at the end of the effective stroke; the first is inhibited by calcium and the second by vanadate or azide. This is consistent with a model of ciliary beating where microtubule doublet numbers 1, 2, 3, and 4 are active during the effective stroke while microtubule doublets numbers 6, 7, 8, and 9 are passive, and the converse occurs during the recovery stroke.

Key words: switch hypothesis, cilia, motility, vanadate, calcium, dynein

Following the identification by Cantley et al [1] of vanadium in the +5 oxidation state (vanadate) as a potent (Na, K)-ATPase inhibitor that does not affect sarcoplasmic reticulum Ca^{2+}-activated ATPase, mitochondrial ATPase (F1), or actomyosin ATPase [2], Gibbons et al [3] and others [4, 5] have shown that micromolar concentrations of vanadate inhibit the Mg^{2+}-activated dynein ATPase of cilia. Particularly, Gibbons et al [3] showed that vanadate inhibits the motility of detergent-treated sea urchin sperm reactivated with ATP. However, at much higher concentrations vanadate is ineffective in arresting untreated sperm, presumably because it does not penetrate the intact sperm cell membrane. Sale and Gibbons [6] showed that vanadate also inhibits the sliding of microtubule doublets of trypsin-treated axonemes.

In this paper we report on the effects of vanadate and other solutions on the motility of in vivo L-cilia of freshwater mussel gills. There are three main types of cilia on the frontal face of freshwater mussel gills, distinguished by their length and arrangement: the frontal (F), laterofrontal (LF), and lateral (L) cilia. There are about 200 cilia per L-cell;

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these cilia beat with metachronal rhythm when perfused with 12.5 mM $CaCl_2$, producing the well-known metachronal waves (MW) with wavelengths of approximately 10 μm traveling along the epithelium at rates of several hundred micrometers per second.

We have found that millimolar concentrations of vanadate inhibit the motility of in vivo L-cilia of freshwater mussel gills. These cilia arrest in a "hands down" position, ie, pointing abfrontally. This is different from the position of L-cilia arrested with calcium and ionophore [7]. Satir found [7] that L-cilia treated with calcium and ionophore arrest in a "hands up" position, ie, pointing frontally. This arrest has been shown by Walter and Satir [8] to be due to an increase of Ca^{2+} around the ciliary axoneme. L-cilia also arrest in a "hands up" position in response to various types of physical and chemical stimulation [9, 10].

Based on the observation that L-cilia can be arrested in one of two unique positions, we suggest that there are two switch points for activation of active sliding in ciliary beat.

MATERIALS AND METHODS

Materials

Sodium orthovanadate (V) – probable formula, $Na_3VO_4 \cdot 16H_2O$ – and ionophore A23187 were obtained from Fisher Scientific Co. and Eli Lilly and Co., Indianapolis, respectively. Vanadate solutions were prepared in reliance on the probable formula weight and are approximate; the ionophore was prepared as a 10^{-3} M stock solution in 0.25% dimethyl formamide and 0.75% ethanol. Freshwater mussels (Margaritana margaritifera and Elliptio complanatus) were obtained from Connecticut Valley Biological Supply Co., Inc. Triton X-100, ATP disodium salt, and DL-dithiothreitol (DTT) were obtained from Sigma Chemical Co.

Methods

Gills of freshwater mussels were stripped while still in the animal; then they were excised and placed in 12.5 mM $CaCl_2$ and 1 mM Tris-HCl, pH 7.4. For experimentation, pieces of stripped gill were placed on microscope slides, where they were gently stretched and held in place. The slide with the specimen was then placed in a Petri dish containing the experimental solution. All solutions used here contained 1 mM Tris-HCl, buffered at pH 7.4. The pH remained unaltered during experimentation. Observations were made with a Zeiss microscope adjusted for bright-field illumination. For detailed examination of the specimens, pieces of gill were quick-fixed [11], embedded in Epon, and sectioned with glass knives to about 2 μm in thickness. The sections were observed and photographed in appropriate orientation under phase-contrast microscopy. The reactivation experiments utilized the following protocols: Incubation of gills in 35 mM KCl and 5 mM phosphate buffer (pH 7.0) at room temperature (25°C) for 3 h produced an en masse exfoliation of motile ciliated cells [8]. The cells were washed and resuspended in 50 mM KCl, 2 mM EGTA, 4 mM $MgSO_4$, 1 mM DTT, and 30 mM HEPES, pH 7.0 (wash solution), and stored in crushed ice. A 20-μl aliquot of cell suspension was mixed with 10 μl of detergent solution (wash solution containing 0.04% w/v Triton X-100 and stored at 0°C) and incubated at room temperature for 15–45 sec. Triton treatment was terminated by the addition of 200 μl of wash solution. This treatment rendered the ciliated cells motionless; perfusion of reactivation solution (wash solution containing 2 mM ATP) across these cells caused them to resume beating. Inhibitors were added to the reactivation solution as indicated.

RESULTS

L-cilia beat with metachronal rhythm with a mean beat frequency of about 17 Hz. Figure 1a shows an instantaneously fixed preparation that preserves the MW on both sides of the gill filaments. Satir [11] has analyzed such preparations and has indicated correspondence between the preserved wavelength and the ciliary beat. Each wavelength contains cilia captured in a variety of stroke positions; these repeat from wavelength to wavelength. Figure 2a is a transverse section through a gill filament where L-cilia were fixed in MW positions. Note that the appearances of the L-cilia on the two sides of the filament are different; actually, portions of several differently positioned cilia are sectioned in each half of the filament. In addition, in such a transverse orientation, different beat positions are also seen from filament to filament. LF and F cilia are also seen in Figure 2a. This appearance is in contrast with the position of L-cilia arrested with 12.5 mM $CaCl_2$ and 10^{-5} M A23187 [7]. In this case all L-cilia stop in a specific position. Figure 1b shows a random longitudinal section of an instantaneously fixed preparation of a gill filament treated with calcium and ionophore. The portions of the L-cilia shown in this figure form a dense mat, in which no MW positional variation can be seen. The section in Figure 1b is taken frontally to the position of the L-cells, suggesting that the cilia point in a frontal direction. This is better seen in Figure 2b, which is a transverse section of the same preparation as Figure 1b. The L-cilia in both sides of the filament are all in the same stroke position, a "hands up" position, corresponding to the end of the recovery stroke.

We have found that within 10 min following treatment of the excised gill epithelium with about 20 mM V, the MW also disappears and the L-cilia again appear to have stopped in one specific position. Figure 1c shows a random longitudinal section of an instantaneously fixed preparation from such an experiment. The L-cilia on both sides of the filament form a dense mat, indicating that the cilia have been stopped. However, this section is taken abfrontally to the L-cells, suggesting that the cilia point in an abfrontal direction, ie, opposite to Figure 1b. This is better seen in Figure 2c, where a random transverse section of a preparation of the same experiment shows that all L-cilia are arrested in the same position, a "hands down" position, corresponding to the end of the effective stroke.

We have been able to switch arrested L-cilia from one arrest position to another without restarting beat. Treatment of L-cilia with 5 mM $CaCl_2$ and 10^{-5} M A23187 causes the cilia to arrest in the "hands up" position, as shown in Figure 3a. Changing this solution to 5 mM $CaCl_2$, 10^{-5} M A23187, and 10 mM V causes the cilia to move and arrest in a neutral position, where the cilia are approximately straight, as shown in Figure 3b. Further replacement by 10 mM V alone causes the cilia to move and arrest in the "hands down" position, as shown in Figure 3c. Switching the arrested position of the L-cilia can also be accomplished in the reverse direction. Treatment of L-cilia with 10 mM V causes the cilia to arrest in a "hands down" position (Fig. 4a). Addition of Ca^{2+} and ionophore causes the cilia to move and arrest at a neutral position (Fig. 4b), where they are approximately straight, and complete substitution causes most of the L-cilia to move to a "hands up" position (Fig. 4c). Not all cilia move to a "hands up" position, presumably because the effect of the vanadate on L-cilia in vivo is not readily reversible when treatment with vanadate is continued for periods of time longer than 10 min.

We wanted to determine whether the movement of L-cilia from one arrested position to the other is energy-dependent. We attempted to do this by treating the gill with solutions containing azide, and the results were somewhat unexpected. Treatment of a gill with 12.5 mM $CaCl_2$ and 1 mM NaN_3 causes L-cilia to arrest in the "hands down" position

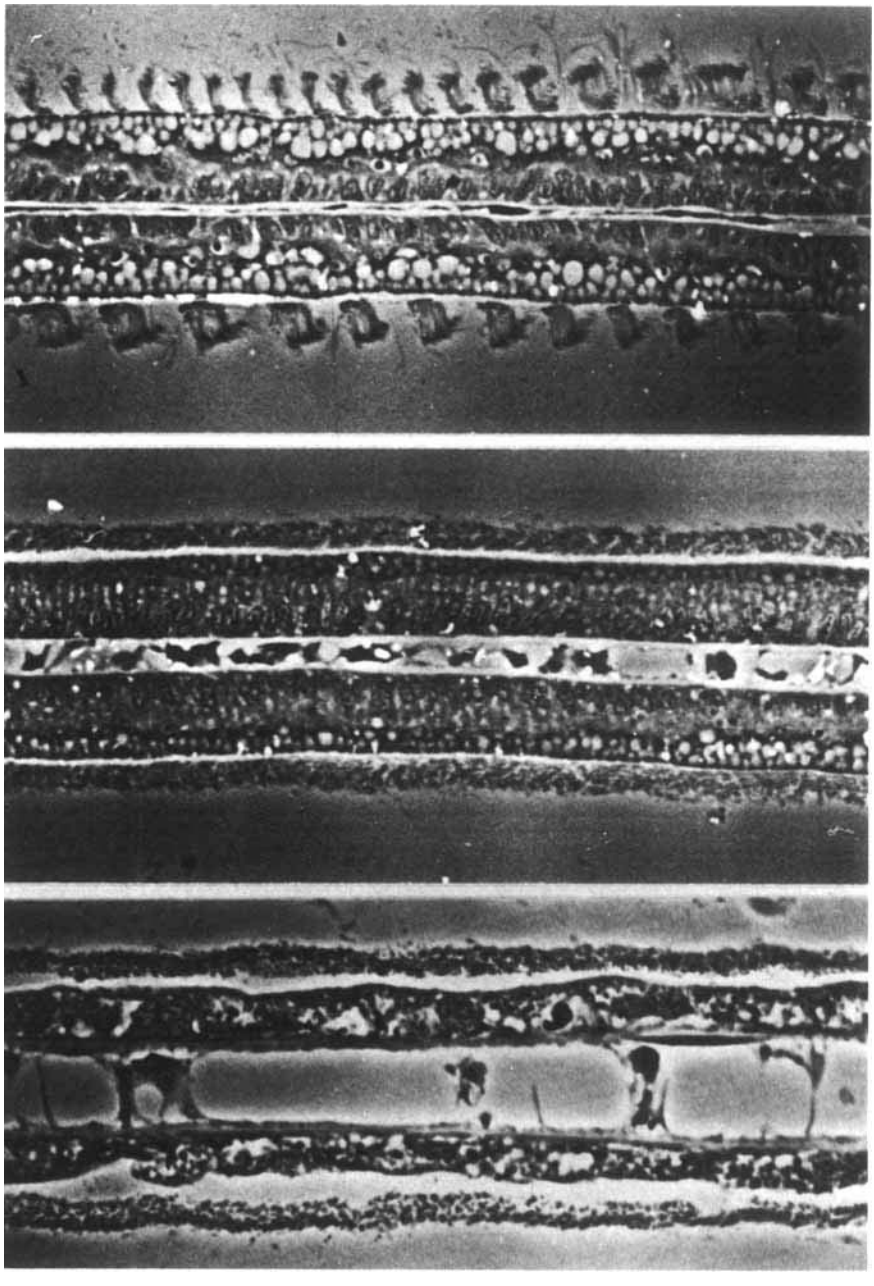

Fig. 1. Phase-contrast micrographs of longitudinal sections of gill filaments after quick fixation. a: Section through the metachronal wave showing L-cilia captured in a wide variety of beat positions; these repeat from wavelength to wavelength. b: Section through a gill filament treated with 12.5 mM $CaCl_2$ and 10^{-5} M A23187; L-cilia arrest and form a dense mat. This section is taken frontally to the position of the L-cells, indicating that the L-cilia point frontally. c: Section through a gill filament treated with 20 mM vanadate; L-cilia also arrest and form a dense mat, but this section is taken abfrontally to the L-cells, indicating that the cilia point abfrontally. × 670.

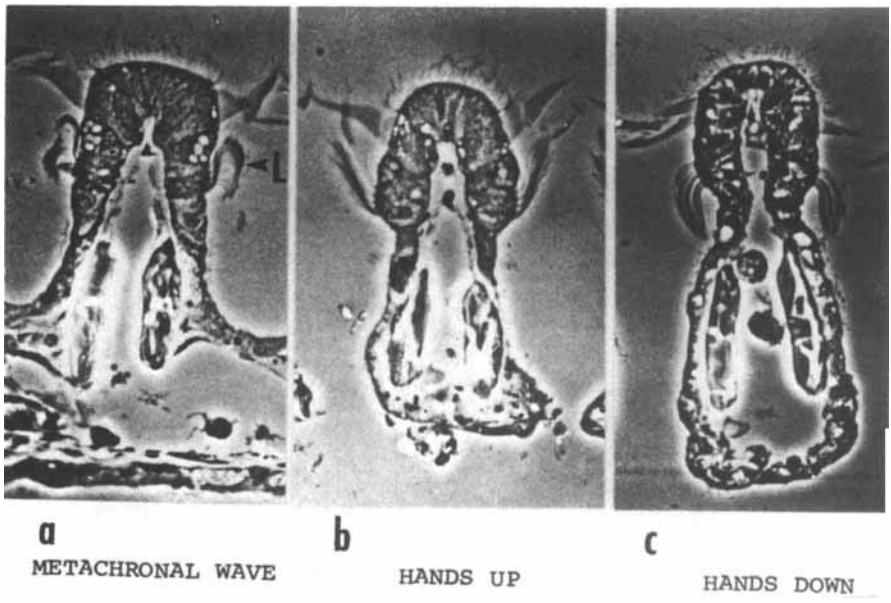

Fig. 2. Phase-contrast micrographs of transverse sections through gill filaments showing L-, LF-, and F-cilia after quick fixation. a: L-cilia fixed in metachronal wave position. b: L-cilia treated with 12.5 mM $CaCl_2$ and 10^{-5} M A23187; cilia arrest in a "hands up" position. c: L-cilia treated with 20 mM vanadate; cilia arrest in a "hands down" position. × 525.

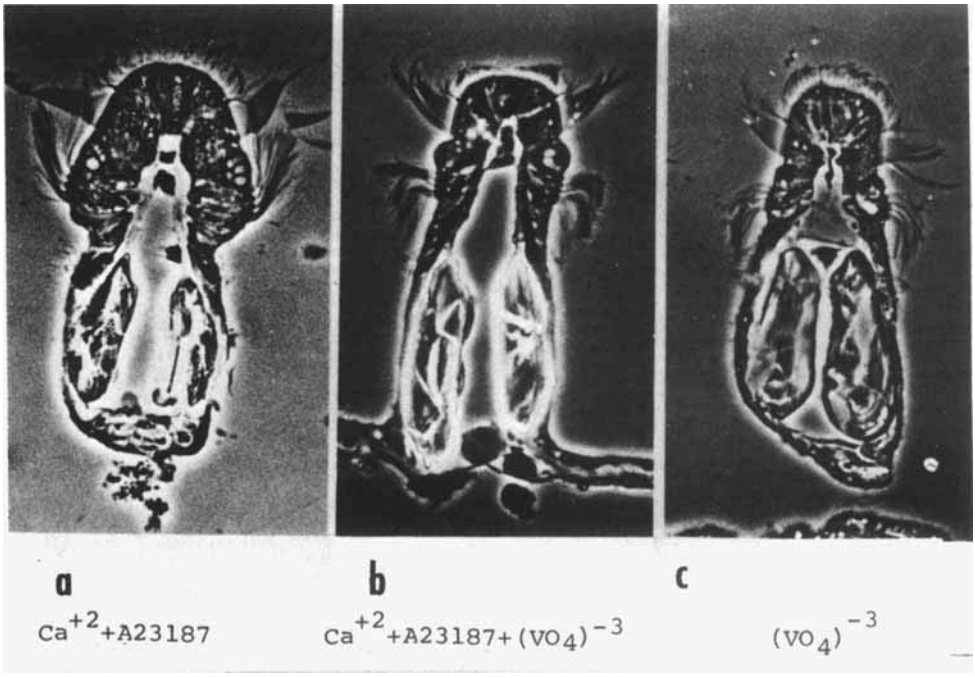

Fig. 3. Phase-contrast micrographs of transverse sections through gill filaments after quick fixation. a: L-cilia treated with 5 mM $CaCl_2$ and 10^{-5} M A23187 arrest in the "hands up" position. b: Addition of 10 mM vanadate to this solution causes cilia to move and arrest at a neutral position. c: Further replacement by 10 mM vanadate alone causes cilia to move and arrest in the "hands down" position. × 525.

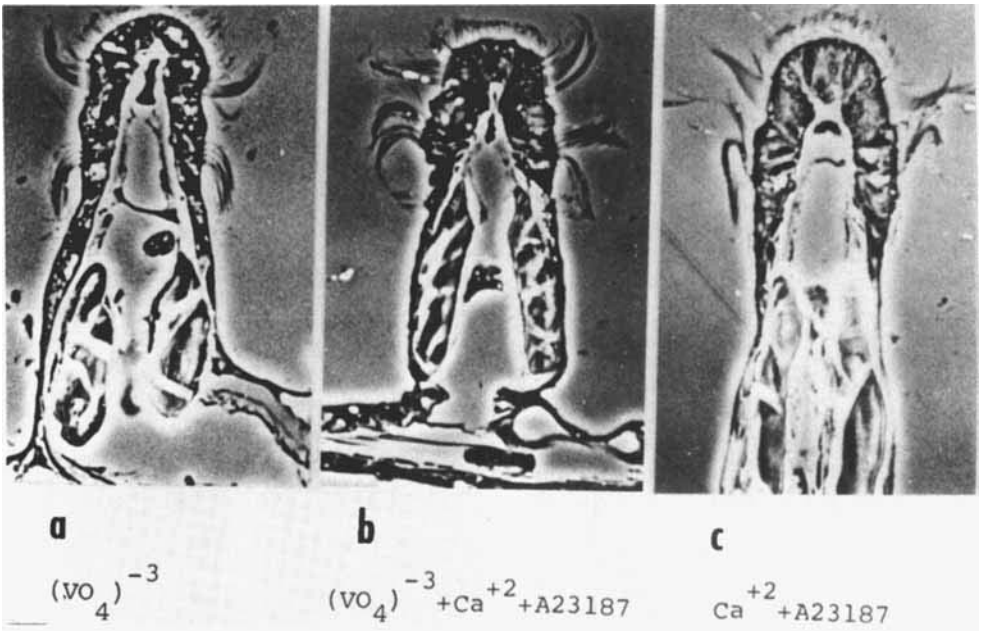

Fig. 4. Phase-contrast micrographs of transverse sections through gill filaments after quick fixation. a: L-cilia treated with 10 mM vanadate arrest in the "hands down" position. b: Addition of Ca^{2+} and ionophore to (a) causes the cilia to move and arrest at a neutral position. c: Complete substitution causes most of the cilia to move and arrest in the "hands up" position. × 525.

as shown in Figure 5a (ie, similarly to vanadate-induced arrest). This suggests that calcium has not entered the cilia. However, within 5 sec of a change of this solution to 20 mM KCl and 1 mM NaN_3 the cilia flick synchronously from the "hands down" position to the "hands up" position, as shown in Figure 5b. After 15 min in KCl and azide or 5 min after return to 12.5 mM $CaCl_2$ and 1 mM NaN_3, the cilia revert to a "hands down" position (Fig. 5c).

If, in the experiment described above, either 20 mM $MgSO_4$, 20 mM NaCl, 25 mM $CaCl_2$, 6 mM $CaCl_2$, or 40 mM sucrose is substituted for 20 mM KCl, essentially no flicking of cilia occurs. When a gill is treated with 1 mM NaN_3 solutions containing 20 mM $MgSO_4$, 20 mM NaCl, or 40 mM sucrose, instead of 12.5 mM $CaCl_2$, arrest is also "hands down," but no flicking of cilia occurs following substitution of these solutions by 20 mM KCl and 1 mM NaN_3. Thus it seems that azide is the agent responsible for "hands down" arrest in these experiments and that a rise in internal Ca^{2+} is needed to cause flicking of cilia. The latter assumption was tested by arresting cilia in a "hands down" position using 12.5 mM $CaCl_2$ and 1 mM NaN_3 and then adding 10^{-5} M A23187. In this experiment the L-cilia moved from a "hands down" position to a "hands up" position in an asynchronous manner and in a longer time (within 15 min) than when treated with 20 mM KCl. The addition of 1 mM $LaCl_3$, a known calcium channel blocking agent, to azide solutions containing Ca^{2+} and K^+ also abolishes flicking of the L-cilia.

We also studied the effect of vanadate and azide on reactivated L-cilia. While 10 mM V was necessary to arrest L-cilia of gills in vivo, reactivated (and presumably demembranated) L-cilia were arrested with 5 μM V. This arrest was readily reversible by washing in reactivation solution free of vanadate. We found that treatment of reactivated L-cilia directly with 1 mM NaN_3 had no effect on their motility. This suggests that the site of action of azide in the in vivo situation is not directly on the axoneme, but presumably is mitochondrial [12], such that respiratory ATP production is blocked or greatly reduced.

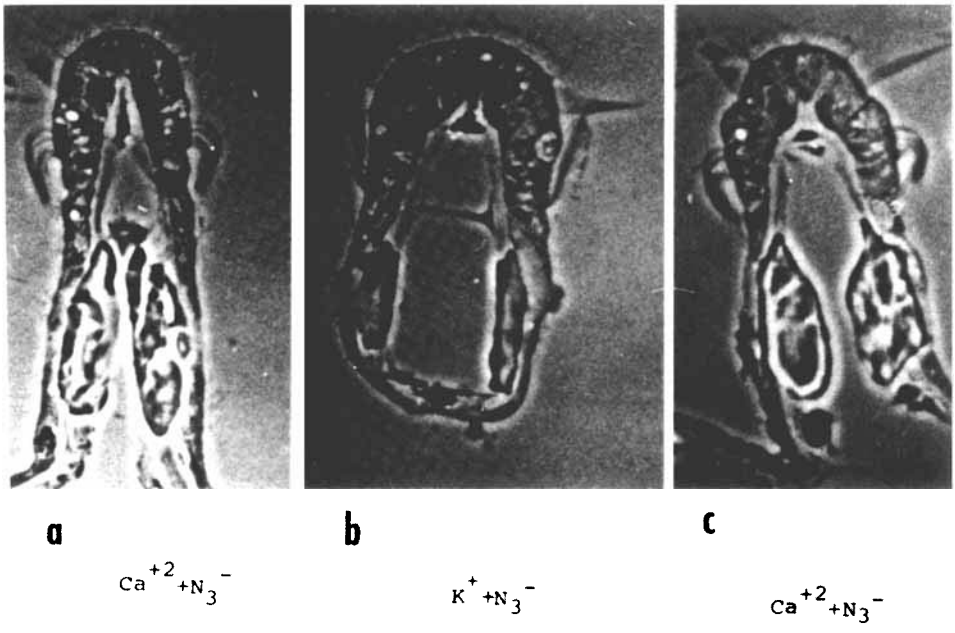

Fig. 5. Phase-contrast micrograph of transverse sections through gill filaments after quick fixation. a: L-cilia treated with 12.5 mM $CaCl_2$ with 1 mM NaN_3 arrest in a "hands down" position. b: Changing of solution to 20 mM KCl and 1 mM NaN_3 causes cilia to flick synchronously to a "hands up" position. c: Returning the gill filament to 12.5 mM $CaCl_2$ and 1 mM NaN_3 causes cilia to revert to a "hands down" position. × 525.

DISCUSSION

L-cilia move through a wide variety of stroke positions during a beat cycle. The observation that these cilia can be made to arrest using the appropriate solution at one of two unique positions, ie, a "hands up" and a "hands down" position, suggests that there are two metastable switch points in a beat cycle; one at the end of the recovery stroke and the other at the end of the effective stroke.

The first arrest position has been described previously [7] and is apparently dependent upon an increase of axonemal free Ca^{2+} to $> 8 \times 10^{-7}$ M [8]. In vivo, such an increase in cytoplasmic Ca^{2+} presumably depends upon depolarization of the cell membrane. This can also be experimentally produced by a divalent cation ionophore (A23187) and high external Ca^{2+}.

The second arrest position has not been described in the recent literature. We have succeeded in producing this arrest either with high external vanadate or with azide. The effects of vanadate and calcium on the motility of L-cilia are directly upon the axoneme. These substances inhibit when applied directly to reactivated cilia in low concentration. Applied externally, the substances must be present in thousand-fold or higher excess, presumably to reach the axonemal concentrations required for inhibition.

Gibbons et al [3] reported that micromolar concentrations of vanadate inhibited the motility of reactivated sea urchin sperm flagella and their Mg^{2+}-activated dynein ATPase. We have found that 5 μm V produces a reversible arrest of reactivated L-cilia. It is then reasonable to assume that the effect of vanadate on the gill cilia is due to its inhibitory effect on the dynein ATPase.

Arrest is produced by 1 mM azide independently of ionic content of the solutions tested, and even in the presence of high external calcium, this arrest is in the "hands down" position. It does not inhibit axonemal reactivation even at relatively high concentrations and therefore does not act directly on the axoneme. Insofar as azide interferes with ATP production it may also indirectly affect the activity of dynein ATPase, lowering sliding velocity to zero. This suggests that the "hands down" position is the passive (low-energy-requiring) ciliary arrest position. In addition, azide alone must be insufficient to open the cell membrane to Ca^{2+}. The presence of K^+, which presumably depolarizes the membrane, or the presence of A23187 produces a change in position (flicking) because Ca^{2+} can enter the cell. In the presence of $LaCl_3$, a Ca^{2+} channel blocking agent, or in the absence of sufficient external Ca^{2+}, the K^+-induced flicking is abolished.

In earlier experiments it was shown that Ca^{2+}-induced arrest was reversible [7]. Here, we have shown that both Ca^{2+}- and V-induced arrest depends on the continued presence of the specific inhibitors and that the arrest positions can readily be switched without restarting beat by substituting one inhibitor for the other. The vanadate-induced arrest is not as easily reversed, presumably because the cells cannot readily pump vanadate out of the axoneme. The presence of two ciliary arrest positions that may be interchanged without restarting beat may provide important clues about the mechanism of doublet microtubule coordination within the axoneme.

Sale and Satir [13] have provided evidence that in trypsin-treated axonemes the microtubule doublets slide actively past each other in only one direction. If this single polarity of active sliding applies to untreated axonemes, as seems likely, it must imply that not all doublets are active at the same time during ciliary beat. The geometry of ciliary bend indicates that during bend generation some of the doublets are sliding towards the tip and others towards the base: Thus, applying the conclusion of Sale and Satir [13] regarding the direction of sliding, we find that only doublets that slide towards the base have active dynein arms, while the others move passively. An analysis of the displacements of adjacent doublets at the end of the recovery and effective strokes by Satir and Sale [14] indicates that doublets numbers 1, 2, 3, and 4 are active while doublets numbers 6, 7, 8, and 9 are passive during the effective stroke. The converse must occur during the recovery stroke. This is illustrated in Figure 6. In L-cilia, doublet No. 5 is always seen attached to doublet 6 and thus is presumably passive at any time. This model predicts that there must be a point during the effective stroke where doublets 1, 2, 3, and 4 are switched off and doublets 6, 7, 8, and 9 are switched on. The converse happens at the end of the recovery stroke.

Our results indicate that the switch point at the end of the effective stroke is vanadate- or azide-sensitive and the switch at the end of the recovery stroke is calcium-sensitive. In the presence of azide or vanadate, L-cilia will not be able to switch from the effective stroke to the recovery stroke and will remain in a "hands down" position. Addition of calcium and ionophore to vanadate-arrested L-cilia causes the cilia to move from the "hands down" position to a neutral position. This suggests that calcium may antagonize the effect of vanadate and allow some of the microtubules that are "on" to be turned "off" and some other microtubules that are "off" to be turned "on." The resulting combination of partially active microtubules moves the cilium. Similarly, in the presence of calcium the L-cilia will not be able to switch from the recovery to the effective stroke and will remain in a "hands up" position. Addition of vanadate to calcium-arrested L-cilia again causes the cilia to move to a neutral position.

Azide, vanadate, and calcium have different effects on ciliary motility. The specificities of these substances suggest that the switch point mechanism at the end of the effective stroke is dependent on the dynein cycle, while the second switch is dependent on

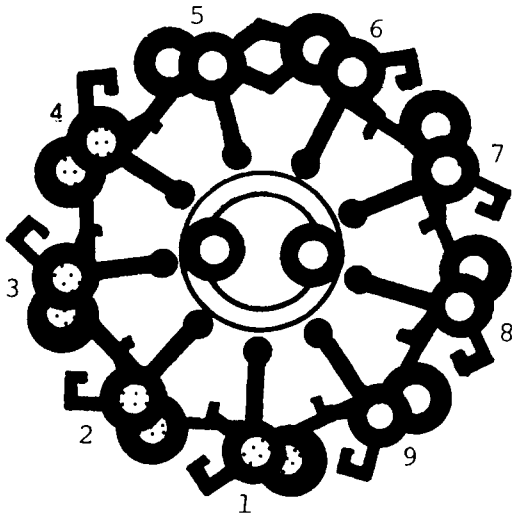

Fig. 6. Diagram of a cross section of an L-cilium axoneme illustrating the mode of operation of doublet microtubules during ciliary beating, as proposed in this paper. In these axonemes doublet 5 is always seen attached to doublet 6 and is thus presumably passive at any time. The stippled doublets are active during the effective stroke, while those not stippled are passive. The activity pattern reverses during the recovery stroke.

a different mechanism, sensitive to calcium. Warner and Satir [15] have proposed that the active sliding of microtubule doublets is converted into local bending by the cyclic attachment and detachment of radial spokes extending from the A microtubule to the projections of the central sheath. This interaction may be the calcium-sensitive mechanism where the second switch point mechanism resides.

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REFERENCES

1. Cantley LC, Josephson L, Warner R, Yanagisawa M, Lechene C, Guidotti G: J Biol Chem 252: 7421, 1977.
2. Josephson L, Cantley LC: Biochemistry 16:4572, 1977.
3. Gibbons IR, Cosson MP, Evans JA, Gibbons BH, Houck B, Martinson KH, Sale WS, Tang WY: Proc Natl Acad Sci USA 75:2220, 1978.
4. Kobayashi T, Martensen T, Nath J, Flavin M: Biochem Biophys Res Commun 81:1313, 1978.
5. Martensen T, Kobayashi T, Nath J, Flavin M: Fed Proc, vol 37, p 2864, 1978 (Abstract).
6. Sale SW, Gibbons IR: J Cell Biol 82:291, 1979.
7. Satir P: Science 190:586, 1975.
8. Walter MF, Satir P: J Cell Biol 79:110, 1978.
9. Murakami M, Takahashi K: Nature 257:48, 1975.
10. Satir P, Reed W, Wolf DI: Nature 263:520, 1976.
11. Satir P: J Cell Biol 18:366, 1963.
12. Hochster RM, Quastel JH: "Metabolic Inhibitors." New York: Academic, 1963, p 395.
13. Sale WS, Satir P: Proc Natl Acad Sci USA 74:2045, 1977.
14. Satir P, Sale WS: J Protozool 24:498, 1977.
15. Warner FD, Satir P: J Cell Biol 63:35, 1974.