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RECONSTITUTION OF METACHRONAL WAVES IN CILIATED CORTICAL SHEETS OF *PARAMECIUM*

II. ASYMMETRY OF THE CILIARY MOVEMENTS

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Summary

In conditions in which ciliated cortical sheets prepared from detergent-extracted *Paramecium multimicronucleatum* cells adhered to glass coverslips on a microscope stage, perfusion of a reactivation medium containing ATP plus cyclic AMP or cyclic GMP generated metachronal waves. An analysis of the ciliary movements that generate these metachronal waves yielded the following results. During the generation of metachronal waves, there were phase differences in the ciliary orientation of adjacent cilia in the direction of wave propagation. Addition of cyclic AMP or cyclic GMP increased the rotational angular velocities during the effective stroke of ciliary beating, but did not increase the rotational angular velocity of the recovery stroke. When the ATP concentration in the cyclic GMP reactivation medium was increased, the rotational angular velocity during the effective stroke rose steeply and saturated at 0.8 mmol l^{-1} ATP, whereas that during the recovery stroke rose gradually. Addition of cyclic nucleotides caused a single cilium isolated from neighbouring cilia on the cortical sheet to incline almost parallel to the cortical surface during the recovery stroke. Addition of cyclic GMP increased the amplitude of bending of cilia detached from the cortical sheet. From these results, it was concluded that increases in the asymmetrical movement of individual cilia, caused by the addition of cyclic nucleotides, create the ciliary interaction that generates the metachronal waves.

Introduction

In a preceding study (Okamoto and Nakaoka, 1994), it was found that metachronal waves are generated on ciliated cortical sheets prepared from detergent-extracted *Paramecium* cells when they are perfused with reactivation media containing cyclic AMP or cyclic GMP. In the present study, we describe changes in the ciliary movements accompanying the generation of these metachronal waves.

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Key words: ciliary movements, asymmetry, *Paramecium multimicronucleatum*, cyclic GMP, metachronal waves.

The study of living *Paramecium* has revealed that, in the cycle of ciliary beating, there are two phases of movement: the effective stroke, in which the cilia move quickly in a plane nearly vertical to the cell surface; and the recovery stroke, in which the cilia move slowly while inclined close to the cell surface (Parducz, 1967; Machemer, 1972). Such asymmetrical movements are coordinated to give a constant phase difference between adjacent cilia, giving rise to the appearance of propagated waves across the ciliary field.

Using ciliated cortical sheets, it is possible to control the generation of metachronal waves. We therefore attempted to detect changes in ciliary movements associated with wave generation. The asymmetrical movements of individual cilia were examined when they were isolated from neighbouring cilia. The asymmetrical movements of individual cilia, caused by the addition of cyclic nucleotides, were found to generate the propagating metachronal waves.

Materials and methods

The preparation of ciliated cortical sheets from *Paramecium multimicronucleatum* cells, their reactivation, observation and video recording have been described previously (Okamoto and Nakaoka, 1994).

Isolation of cilia and reactivation

For deciliation, Triton-extracted cells were suspended in a solution containing (in $mmol\,l^{-1}$): KCl, 20; $CaCl_2$, 2; $MgCl_2$,1; Tris–maleate, 10; pH 7.0 at 0 °C for 3 min. After low-speed centrifugation to remove the cell body, the supernatant was further centrifuged at 10 000 ***g*** for 10 min. The ciliary pellet was suspended in a solution containing (in $mmol\,l^{-1}$): KCl, 20; $MgCl_2$, 1; Tris–maleate, 10 (pH 7.0) and this suspension was incubated at 0 °C.

Reactivations were made by suspending the cilia in the basic reactivation medium, in 10 $\mu mol\,l^{-1}$ cyclic ATP reactivation medium or in 100 $\mu mol\,l^{-1}$ cyclic GMP reactivation medium (Okamoto and Nakaoka, 1994).

Analyses of ciliary movements

The frequency of ciliary beating was determined by counting ciliary base rotations. Effective and recovery strokes were distinguished by the difference in the angular velocities during the ciliary cycle.

For analysis of the movement of a single cilium, dark-field video images recorded every 1/60 s were successively summed into a single image. In the case of cilia on a cortical sheet, successive video images were digitally stored in a computer (Macintosh IIci, Apple), and the images of the ciliary part were transferred into a single frame using software (Photoshop, Adobe) that adjusted non-motile points that were common to each frame. For a cilium detached from the cortical sheet, the video images displayed on a monitor screen were photographed with a 1 s exposure.

Results

Orientation of cilia on a ciliary field

Video frames of the reactivated cilia on the cortical sheet were displayed on the monitor screen, and instantaneous distributions of ciliary orientation were compared in the basic, cyclic AMP and cyclic GMP reactivation media (Fig. 1).

Orientations of cilia in the basic reactivation medium, in which no propagating waves were produced, showed wide variation, with most cilia pointing in a similar direction (Fig. 1A). A few frames later (not shown), the distribution changed in phase so that all the cilia pointed in another direction. In the basic reactivation medium, a

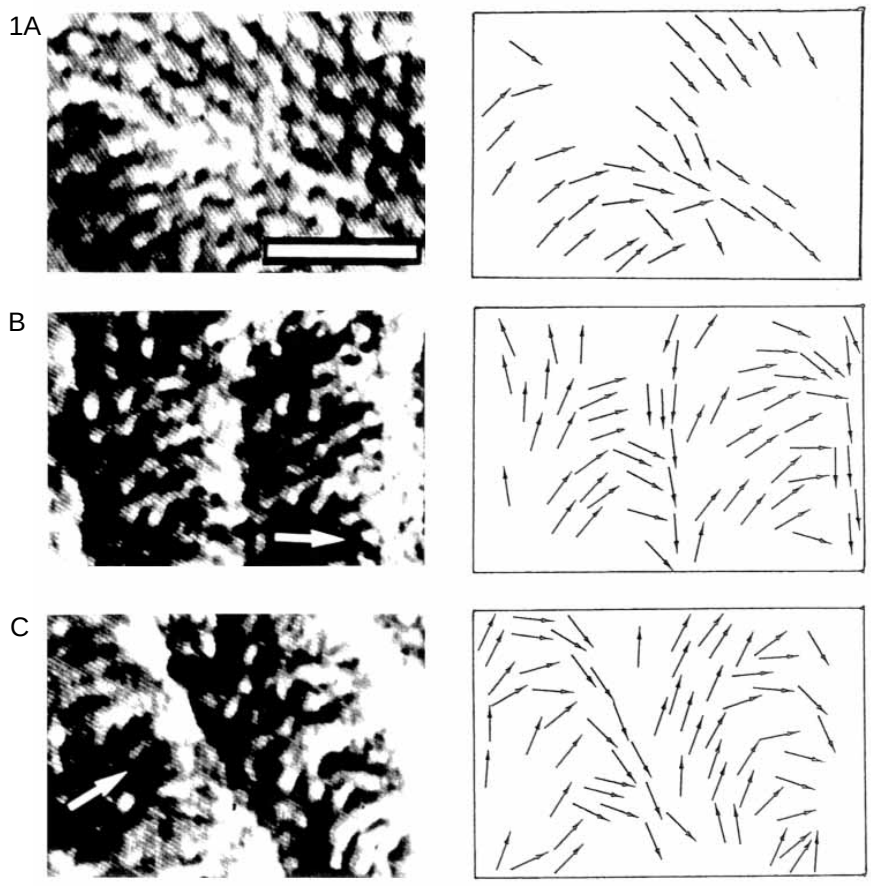

Fig. 1. Distribution of ciliary orientations on the cortical sheet. A cortical sheet was reactivated successively by the basic reactivation medium (A), 10 μmol l^{-1} cyclic AMP reactivation medium (B) and 100 μmol l^{-1} cyclic GMP reactivation medium (C) and observed on a dark-field microscope. Photographs were taken from video frames recorded 10–20 s after perfusion of the respective reactivation medium. The anterior end of the cell is at the top. The open arrows in the photographs show the direction of wave propagation. Arrows in the figure on the right indicate the orientations of the ciliary tips in the photographs on the left. Scale bar, 10 μm.

large proportion of the cilia stood up on the sheet and their orientation could not be determined.

In the cyclic AMP or cyclic GMP reactivation media, in which propagating waves were produced, cilia on the wave crest were oriented along the wave crest line (Fig. 1B,C). This orientation represented the end of the effective stroke of the ciliary cycle. Phase differences in the ciliary cycle were apparent between adjacent cilia in the direction of wave propagation, which agrees with the previous observations of living *Paramecium* (Parducz, 1967; Machemer, 1972).

Rotational angular velocity of the ciliary base

When ciliary movement was observed from above the ciliated surface, the ciliary base was seen to rotate counterclockwise. It moved quickly during the effective stroke and slowly during the recovery stroke. We measured the effects of cyclic AMP and cyclic GMP on this ciliary rotation.

In the basic reactivation medium, in which metachronal waves were absent, the

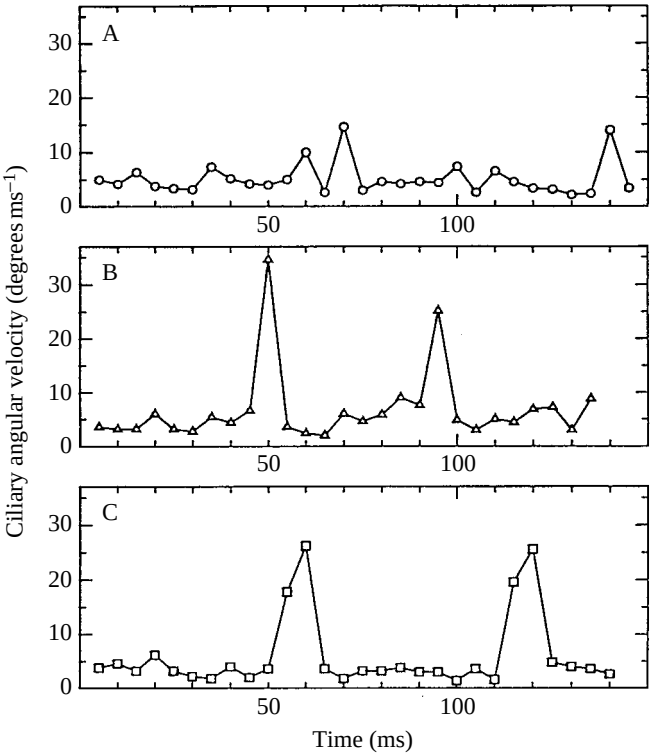

Fig. 2. Angular velocities of ciliary movements on the cortical sheet. Ciliary movement was reactivated successively by the basic medium (A), 10 μmol l^{-1} cyclic AMP medium (B) and 100 μmol l^{-1} cyclic GMP medium (C). Angular changes in the ciliary base orientation were measured every 5 ms (frame-to-frame). Typical data from 10–12 cycles of ciliary beating in the respective conditions are plotted for approximately two cycles.

rotational angular velocities during effective and recovery strokes were, respectively, 12±2 degrees ms^{-1} (N=10) and 5±2 degrees ms^{-1} (N=70) (Fig. 2A). The frequency of ciliary beating was 17±3 Hz (N=10).

When metachronal waves were generated during perfusion of cyclic AMP reactivation medium, the angular velocities during the effective and recovery strokes were, respectively, 30±4 degrees ms^{-1} (N=10) and 5±2 degrees ms^{-1} (N=50) (Fig. 2B). The frequency of ciliary beating was 22±2 Hz (N=10). In the cyclic GMP reactivation medium, the angular velocities during effective and recovery strokes were, respectively, 22±4 degrees ms^{-1} (N=8) and 3±1 degrees ms^{-1} (N=46) (Fig. 2C). In this case, the frequency of ciliary beating was approximately 19±3 Hz (N=10).

It is apparent that, when metachronal waves were created in the cyclic AMP and cyclic GMP reactivation media, the angular velocities during the effective stroke were, respectively, 2.5-fold and 1.8-fold greater than that in the basic reactivation medium. The angular velocities during the recovery stroke were almost the same under all three conditions.

Dependence of ciliary movements on the concentration of ATP

Ciliary movements reactivated by the cyclic GMP medium showed clear differences between the effective and recovery strokes, and the pattern of metachronal waves corresponded to that of living cells swimming forwards (Okamoto and Nakaoka, 1994). We examined the dependencies of the rotational angular velocities during the effective and recovery strokes on the ATP concentration of the cyclic GMP reactivation medium.

The rotational angular velocity during the effective stroke rose steeply with an increase in ATP concentration and saturated at approximately 0.8 mmol l^{-1} (Fig. 3A). In contrast,

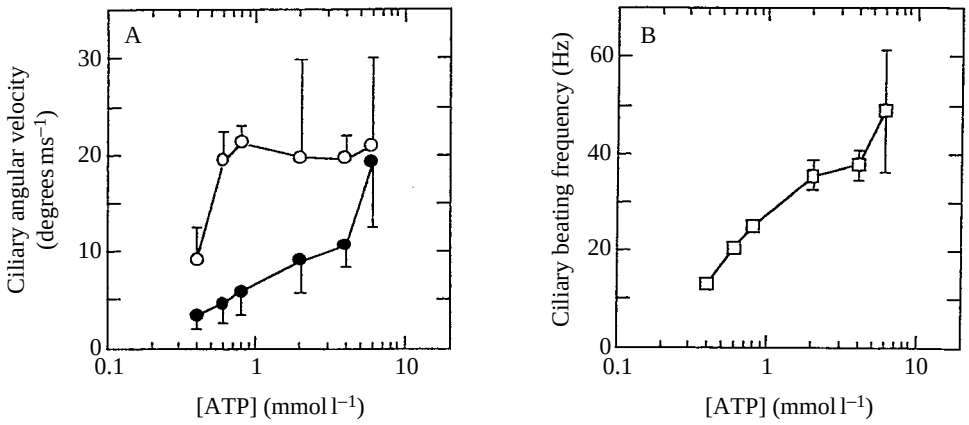

Fig. 3. Dependence of the angular velocity (A) and the ciliary beating frequency (B) on the ATP concentration. The ciliated cortical sheet was reactivated by 100 μmol l^{-1} cyclic GMP media containing different concentrations of ATP. Open circles, ciliary angular velocities during the effective stroke; filled circles, ciliary angular velocities during the recovery stroke; open squares, ciliary beating frequency. Values are means ± S.D. (N=4–20).

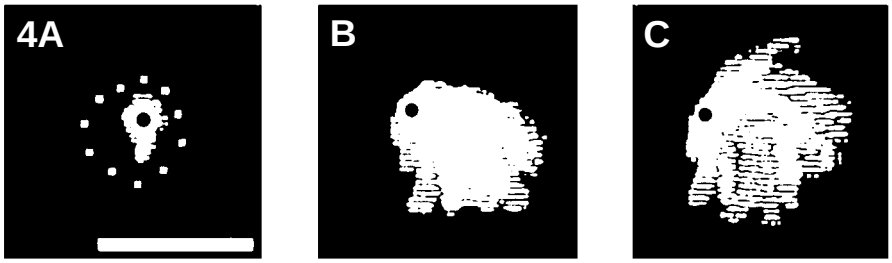

Fig. 4. Effects of cyclic AMP and cyclic GMP on the form of beating exhibited by isolated cilia. A single cilium on a small cortical sheet was reactivated successively by basic medium (A), 10 μmol l^{-1} cyclic AMP medium (B) and 100 μmol l^{-1} cyclic GMP medium (C). In each medium, 24 successive images taken at 1/60 s intervals were summed into a single image using computer analysis. The white area shows the extent of ciliary movement. The black spot shows the position of the ciliary base, and the ring of white spots in A shows the area of the cortical sheet. Scale bar, 10 μm.

the angular velocity during the recovery stroke rose only gradually with the ATP concentration.

The frequency of ciliary beating increased gradually with ATP concentration (Fig. 3B), in parallel with the increase in angular velocity during the recovery stroke. The propagation velocity of the metachronal waves rose in proportion to the increase in the beating frequency, keeping the wavelength at approximately 11 μm and the direction of wave propagation at approximately 140 $^{\circ}$ (Okamoto and Nakaoka, 1994).

Effects of cyclic AMP and cyclic GMP on movements of an isolated cilium

In order to test whether the movements of a single cilium, isolated from neighbouring cilia on the cortical sheet, are affected by cyclic AMP or cyclic GMP, the cortical sheet was mechanically disrupted by gentle pipetting. A small piece of cortical sheet containing a single cilium was attached to a coverslip and successively reactivated by perfusions of the reactivation media. During perfusion of the basic reactivation medium, the cilium moved without a large inclination. It was therefore impossible to bring the full cilium length into focus, so it was recorded as a small circle in dark-field images (Fig. 4A). In the cyclic AMP or cyclic GMP reactivation media, the cilium inclined and moved almost parallel to the surface of the cortical sheet during the recovery phase and the radius of the area swept by the cilium extended to approximately 10 μm (Fig. 4B,C). The area swept by the cilium in the cyclic GMP reactivation medium was larger than that in the cyclic AMP medium. At the beginning of the effective stroke, the cilium was perpendicular to the cortical surface and was no longer in focus.

Movements of detached cilia

In order to determine whether movements of cilia detached from the cortical sheet are affected by cyclic GMP, we attempted to reactivate bending movements in cilia detached from the detergent-extracted cells. Although most of the detached cilia did not move, a few of the cilia that had a spherical particle of 1–2 μm diameter at one end began bending

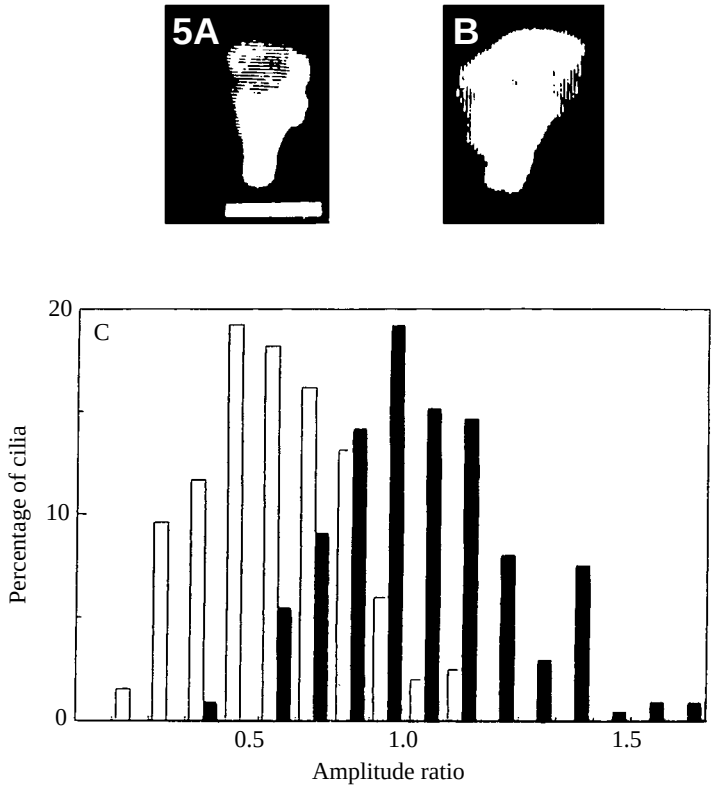

Fig. 5. Changes in the form of beating of isolated cilia. The cilia were detached from the cortical sheet. After suspension in the basic reactivation medium (A) or in the 100 μmol l^{-1} cyclic GMP reactivation medium (B), the swinging movements of cilia that had adhered by one end to the glass surface were video recorded. Sixty successive frames taken at 1/60 s intervals were summed into one photograph. A and B show typical examples of beating cilia. Scale bar, 5 μm. From these photographs, the ratios of the swinging amplitude (linear distance between tip ends) to the ciliary length (linear distance from ciliary base to tip) were obtained. These ratios are shown in the histogram (C) as the percentage of 200 measured cilia reactivated either by the basic medium (open bars) or by the cyclic GMP medium (filled bars).

movements in the cyclic GMP reactivation medium. When a cilium adhered to the coverslip by the spherical particle, it swung its other end around in a funnel-shaped movement. Observed from the upper side, the area swept by the cilium resembled a folded fan (Fig. 5A,B).

The mean amplitude of movement in the cyclic GMP reactivation medium was 1.7-fold larger than that in the basic reactivation medium (Fig. 5C), suggesting that, even after detachment from the cortical sheet, ciliary movements are affected by cyclic GMP.

Discussion

Using the ciliated cortical sheet, it was possible to change the reactivation medium and

to make close microscopic observations of the resulting ciliary movements. The asymmetry in ciliary movements was increased by perfusion of either cyclic AMP or cyclic GMP reactivation media (in which metachronal waves were generated). In terms of temporal asymmetry, the differences in rotational angular velocities between the effective and recovery strokes increased (Fig. 2) and, in terms of spatial asymmetry, the ciliary inclination during the recovery stroke became larger than that in the basic reactivation medium (Fig. 4).

Spatial asymmetry seems to be more important than temporal asymmetry for the metachronal coordination of cilia. Even when the angular velocity during the recovery stroke approaches that during the power stroke at an increased ATP concentration in the cyclic GMP reactivation medium (Fig. 3A), the metachronal waves are still generated. In contrast, a small inclination during the recovery stroke in the basic reactivation medium is not accompanied by metachronal waves. Ciliary inclination during the recovery stroke is, therefore, needed for the generation of metachronal waves. Considering that the distance between cilia on the cortical sheet is 2.5 μm and the inclined cilia extend approximately 10 μm, ciliary interactions that coordinate the metachronal waves are made during the inclined recovery stroke.

In single cilia, isolated from neighbouring cilia, a large inclination during the recovery stroke was induced by the addition of either cyclic AMP or cyclic GMP (Fig. 4). This suggests that the ciliary inclination occurs in each cilium without mechanical interactions between cilia. The experiments using cilia detached from a cortical sheet (Fig. 5) support this view. The increase in the amplitude of swinging induced by cyclic GMP possibly corresponds to the increase in inclination during the recovery stroke, although the nature of the motion of detached cilia must be studied further.

In this study, the dependence on ATP of the ciliary angular velocities during the effective and recovery strokes has been measured separately for the first time. The angular velocity during the effective stroke rose steeply with ATP concentration and saturated at approximately 0.8 mmol l^{-1}, whereas the angular velocity during the recovery stroke increased gradually up to 6 mmol l^{-1} ATP (Fig. 3A). This difference suggests that different ATPases underlie the effective and recovery strokes. In the reactivation of sea-urchin sperm axoneme, both the sliding velocity and the ATPase saturate at approximately 1 mmol l^{-1} ATP (Yano and Miki-Noumura, 1980). Similar saturation is observed in assays of microtubule sliding on ciliary-dynein-coated glass *in vitro* (Vale and Yano-Toyoshima, 1988). The saturation of *Paramecium* ciliary angular velocity during the effective stroke probably corresponds to axonemal sliding and ATPase activity. In contrast, the frequency of the beating of sperm flagella shows a gradual increase with ATP concentration that obeys Michaelis–Menten type kinetics (Gibbons and Gibbons, 1972; Brokaw, 1975). A gradual increase in the beating frequency of detergent-extracted *Paramecium* cilia has previously been reported (Nakaoka *et al.* 1984). Because the rate-limiting step of the ciliary cycle is the slow recovery stroke, the gradual increase in angular velocity during the recovery stroke coincides with the gradual increase in the frequency of beating.

The present study shows that cyclic nucleotides cause different effects on the effective and the recovery strokes: the effective stroke becomes faster and the recovery stroke

inclines closer to the cell surface. Recently, Hamasaki *et al.* (1991) have reported that a cyclic-AMP-dependent phosphorylation of *Paramecium* dyneins increases the sliding velocity of microtubules. We have observed a similar phenomenon in the sliding of *Paramecium* axonemes: the sliding velocity of detergent- and protease-treated ciliary axonemes increased after the addition of either cyclic AMP or cyclic GMP by up to 1.5–1.7 times that in the basic reactivation medium (K. Okamoto and Y. Nakaoka, unpublished data). These results correspond to the observed increase in the angular velocity during the effective stroke. Corresponding data for the recovery stroke are not available. Because the present study implicates the importance of inclination during the recovery stroke, a detailed study of the recovery stroke is needed to elucidate the ciliary interaction that underlies the generation of metachronal waves.

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