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

I. WAVE STABILITIES

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Summary

We reconstituted metachronal waves on ciliated cortical sheets prepared from detergent-extracted *Paramecium multimicronucleatum* cells. Ciliary movements of the cortical sheet, whose intracellular side adhered to a glass coverslip, were reactivated by perfusion of a basic reactivation medium containing ATP. In this condition, the ciliary field showed only unstable localised ripples. Addition of either cyclic AMP or cyclic GMP to the basic reactivation medium generated propagating metachronal waves characteristic of each nucleotide. In order to estimate the stability of the metachronal waves, autocorrelation coefficients were calculated from images of an 8 μm diameter region within the reactivated ciliary field. The decay time for the correlation coefficient to decrease to 0.5 was only 0.04 s in the basic reactivation medium, but was increased to 0.4 or 0.9 s by the addition of cyclic AMP or cyclic GMP, respectively. The decay time was dependent not only on the concentration of cyclic nucleotide but also on the wave frequency. In order to test whether cyclic-nucleotide-dependent phosphorylation affected the generation of waves, the ciliated cortical sheets were thiophosphorylated by incubation in ATP-γ-S (adenosine-5′-*o*-3-thiotriphosphate) medium containing either cyclic AMP or cyclic GMP. Following this, perfusion with the basic reactivation medium generated metachronal waves only after cyclic GMP treatment. The effect of cyclic GMP is probably related to phosphorylation of ciliary proteins.

Introduction

In a population of cilia, ciliary movements are coordinated so as to maintain a constant phase difference between adjacent cilia. The ciliated surface therefore appears to propagate a wave, which is called the metachronal wave. It is now accepted that metachronal coordination is due to mechanical or hydrodynamic forces acting between cilia (Verworn, 1890; Gray, 1930; Sleigh, 1966; Machemer, 1972).

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A ciliated protozoan, *Paramecium multimicronucleatum*, has been used for the study of metachronal coordination (Machemer, 1974). When the cells swim forwards, metachronal waves proceed from the posterior left side to the anterior right side, encircling the cell in left-hand helices of wave crests. The ciliary force during the effective stroke is directed parallel to the wave crests (Parducz, 1967; Machemer, 1972).

When the cell membrane of *Paramecium* is extracted with a detergent, the ciliary movements underlying the metachronal wave stop. They can then be reactivated by suspending the membrane in a defined medium containing ATP (Naitoh and Kaneko, 1972, 1973). It has been shown that the swimming velocity of the reactivated cell models is increased by the addition of either cyclic AMP or cyclic GMP (Nakaoka and Ooi, 1985; Bonini *et al.* 1986; Bonini and Nelson, 1988).

In our study, ciliated cortical sheets were prepared from the extracted cells in order to make a close observation of the ciliary field. When the ciliary movements of the cortical sheets were reactivated by perfusion of an ATP-containing medium, no propagating waves were observed. It was found, however, that a reactivation medium containing either cyclic AMP or cyclic GMP generated propagating metachronal waves. The stability of the wave, which was estimated from autocorrelation of time-dependent changes in the ciliary image, was different in sheets treated with cyclic AMP or cyclic GMP and depended on the ATP concentration. On the basis of these results, we discuss conditions that permit the generation of metachronal waves and the stability of the resultant waves.

Materials and methods

Cells

In order to observe a large area of cortical sheet, we used *Paramecium multimicronucleatum*, which has a large cell size. *Paramecium* were cultured at 25 ˚C in a hay infusion inoculated with *Klebsiella pneumoniae*. Stationary-phase *Paramecium* cells were collected by low-speed centrifugation.

Preparation of ciliated cortical sheets

The preparation of Triton-extracted cells was identical to that used by Nakaoka *et al.* (1984). The extracted cells were suspended in a washing solution containing 20 mmol l^{-1} KCl and 10 mmol l^{-1} Tris–maleate buffer (pH 7.0) for more than 20 min at 0 ˚C. A drop of this suspension was placed on a glass slide, then covered with a coverslip whose edges were coated with Vaseline. During this treatment, the intracellular side of the torn cell cortex adhered to the glass surface of the coverslip, as described by Noguchi *et al.* (1991).

Reactivation

The movements of cilia on the cortical sheets were reactivated by perfusion of reactivation medium from either side of the coverslip. The basic reactivation medium contained (in mmol l^{-1}): ATP, 1; KCl, 4; $MgCl_2$, 4; EGTA, 1; and Tris–maleate buffer, 10 (pH 7.0). For the cyclic AMP or cyclic GMP reactivation media, 10 μmol l^{-1} cyclic AMP or 100 μmol l^{-1} cyclic GMP, respectively, was added to the basic reactivation medium. In the measurements of cyclic AMP or cyclic GMP dependence, the

concentrations of cyclic AMP or cyclic GMP in the reactivation media were changed. ATP-γ-S medium contained 1 mmol l^{-1} ATP-γ-S instead of ATP in the basic reactivation medium. A series of measurements was made on a single sheet by successive perfusions of different media. All of the reactivations were carried out at room temperature (approximately 22 °C).

Observations and recordings

Cilia of the cell surface involved in cell swimming (except the peristomal portion) were observed under a phase-contrast or dark-field microscope. The microscope images were recorded with a high-speed video system (200 frames s^{-1}: MHS-200, NAC) and viewed on the monitor screen of a normal-speed video system (60 frames s^{-1}: AG-3545, Panasonic). The video images were sometimes enhanced with an image processor (Image Sigma-2, Avionics).

The direction of wave propagation was determined as an angle based on the row of ciliary bases arranged parallel to the long axis of the cell. Observing from the upper side of the ciliated surface, the direction of the posterior end was defined as 0 °, moving counterclockwise to the anterior end at 180 °.

Analysis of the ciliary image

Images of the ciliary field were displayed on a monitor screen. The brightness of the ciliary images over an area equivalent to 8 μm in diameter was measured by a photodiode (S2386-18L, Hamamatsu Photonics) positioned on the screen. The diameter for measurements was determined from the experimental observation that the wavelength of the reconstituted metachronal waves was approximately 10 μm. The electric current output of the photodiode was converted to a voltage and band-passed through an active filter whose lower and upper cut-off frequencies were 1 and 20 Hz, respectively. Autocorrelation coefficients and the power spectrum of the voltage output were calculated using a Fast Fourier Transform (FFT) analyzer (AD3524, A&D). In order to obtain cross correlation between two positions within a ciliary field, a pair of the photodiodes was placed on the monitor screen and the voltage signals, obtained as just described, were calculated by the FFT analyzer. In all calculations, sampling time was set to 40 s.

Results

Conditions for generation of metachronal waves

When a ciliated cortical sheet was reactivated by perfusion with the basic reactivation medium, the ciliary field showed some ripples, suggesting the presence of ciliary interactions over short distances. These ripples were unstable and never propagated (Fig. 1A). Fluctuations in the brightness of the ciliary field showed little regularity (Fig. 1A, lower trace). No clear propagating waves were observed in the basic reactivation medium.

When the cyclic AMP reactivation medium was perfused, propagating waves appeared (Fig. 1B). In this case, the direction of wave propagation was 84±15 ° (N=5), directed transversely to the right (anatomical left of the organism), and the wavelength was

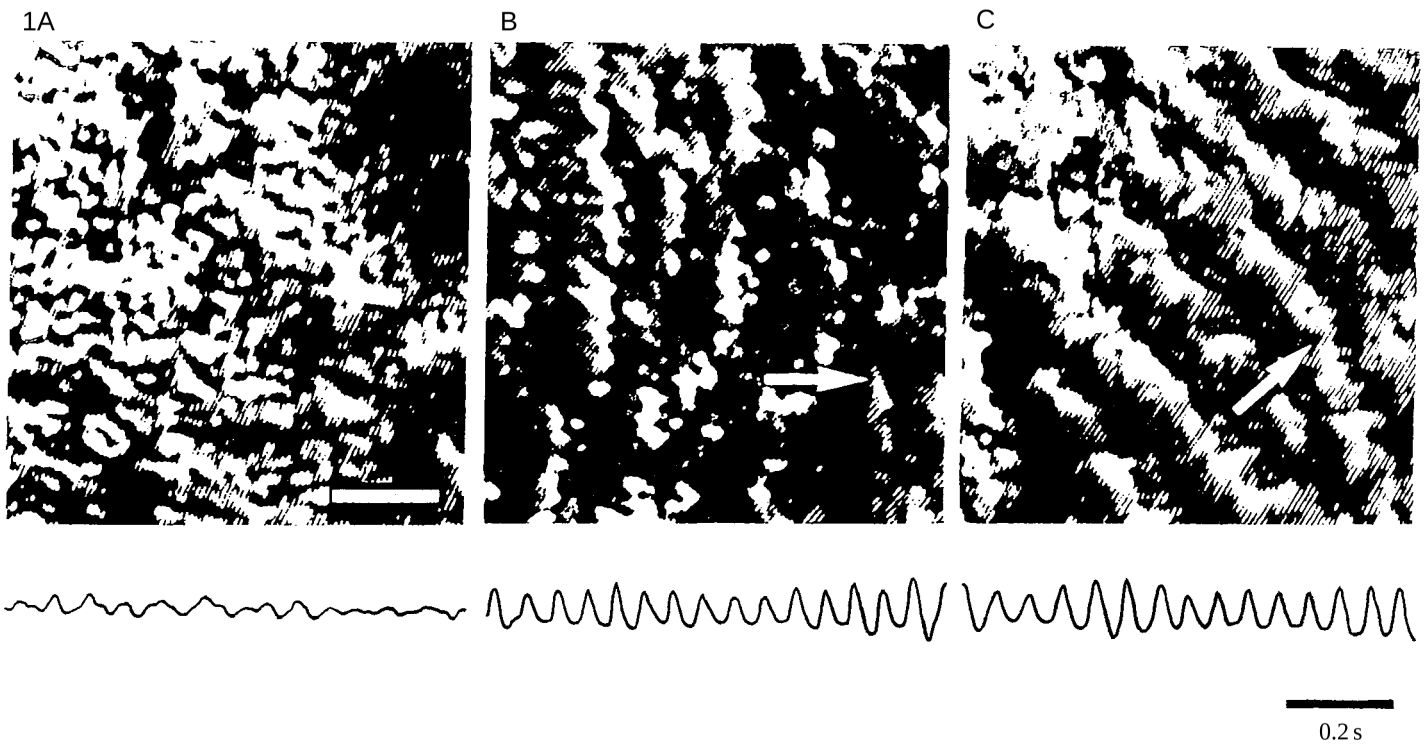

Fig. 1. Metachronal waves reconstituted in a ciliated cortical sheet from *Paramecium multimicronucleatum*. Photographs of the ciliary field and accompanying photocurrents (lower trace) were taken from the video monitor 10–20 s after perfusion of basic reactivation medium (A), 10 μmol l^{-1} cyclic AMP reactivation medium (B) or 100 μmol l^{-1} cyclic GMP reactivation medium (C). These photographs show the same cortical sheet. Arrows in B and C indicate the direction of wave propagation. The top of the photograph is the anterior end of the cell. Scale bar, 15 μm.

10.1±1.7 μm (N=10). The brightness of the ciliary field showed clear cyclical changes accompanying the beat (Fig. 1B, lower trace).

When the ciliated cortical sheet was reactivated with the cyclic GMP reactivation medium, propagating waves were again generated (Fig. 1C). The direction of wave propagation was 136±16.4 ° (N=5), directed towards the anterior right (anatomical left of the organism), and the wavelength was 11.7±1.4 μm (N=10). The brightness of the ciliary field also showed cyclical changes with the beat (Fig. 1C, lower trace).

After testing either the cyclic AMP or cyclic GMP reactivation medium, perfusion of the basic reactivation medium caused the propagating waves to disappear. Subsequent perfusion with the reactivation medium containing cyclic nucleotides again generated each type of propagating wave.

Autocorrelations and power spectra of the metachronal waves

In order to estimate the stability of reactivated metachronal waves, an autocorrelation coefficient and a power spectrum were calculated for the ciliary field. The autocorrelation coefficient is an ensemble mean of the product of brightness fluctuation at time zero and after an arbitrary time interval and is a measure of the similarity of the brightness fluctuations at different times. In the basic reactivation medium, the autocorrelation coefficient decreased rapidly with time (Fig. 2Ai), but in the presence of cyclic nucleotides, decay of the coefficients was much slower (Fig. 2Aii,iii). As a measure of the wave stability, we adopted the decay time for the coefficient to decrease to 0.5.

The power spectrum is the spectrum density of the brightness changes in the ciliary field. The power spectrum in the presence of cyclic nucleotides showed a peak at the mean of the wave frequency (Fig. 2Bii,iii). In the basic reactivation medium, there was no clear peak (Fig. 2Bi). The wave frequencies in the presence of cyclic nucleotides were subsequently determined from the peak of the power spectrum.

When the cyclic AMP concentration in the reactivation medium was increased stepwise, the decay time increased steeply, attained a maximum at 10 μmol l^{-1}, then decreased gradually (Fig. 2Ci). The wave frequency rose monotonically with the increase in cyclic AMP concentration to approximately 150 % of the frequency in the basic reactivation medium (Fig. 2Di). Propagating waves were usually generated at all cyclic AMP concentrations within the range examined (5–100 μmol l^{-1}). The direction of propagation was 80–100 ° and the wavelength was approximately 10 μm.

With an increase of cyclic GMP concentration in the reactivation medium, the decay time rose, attained a maximum at 100 μmol l^{-1}, then dropped gradually (Fig. 2Cii). The peak value of the decay time was about three times longer than that for cyclic AMP treatment. The wave frequency rose with the cyclic GMP concentration to approximately 140 % of that in the basic reactivation medium (Fig. 2Dii). The propagating waves were always produced in the range from 50 μmol l^{-1} to 1 mmol l^{-1} cyclic GMP, but were not produced below 50 μmol l^{-1} cyclic GMP. The direction of wave propagation ranged from 110 to 160 °, and the wavelength was approximately 11 μm.

Dependence on wave frequency

We attempted to compare the stability of waves in the ciliated cortical sheet with those

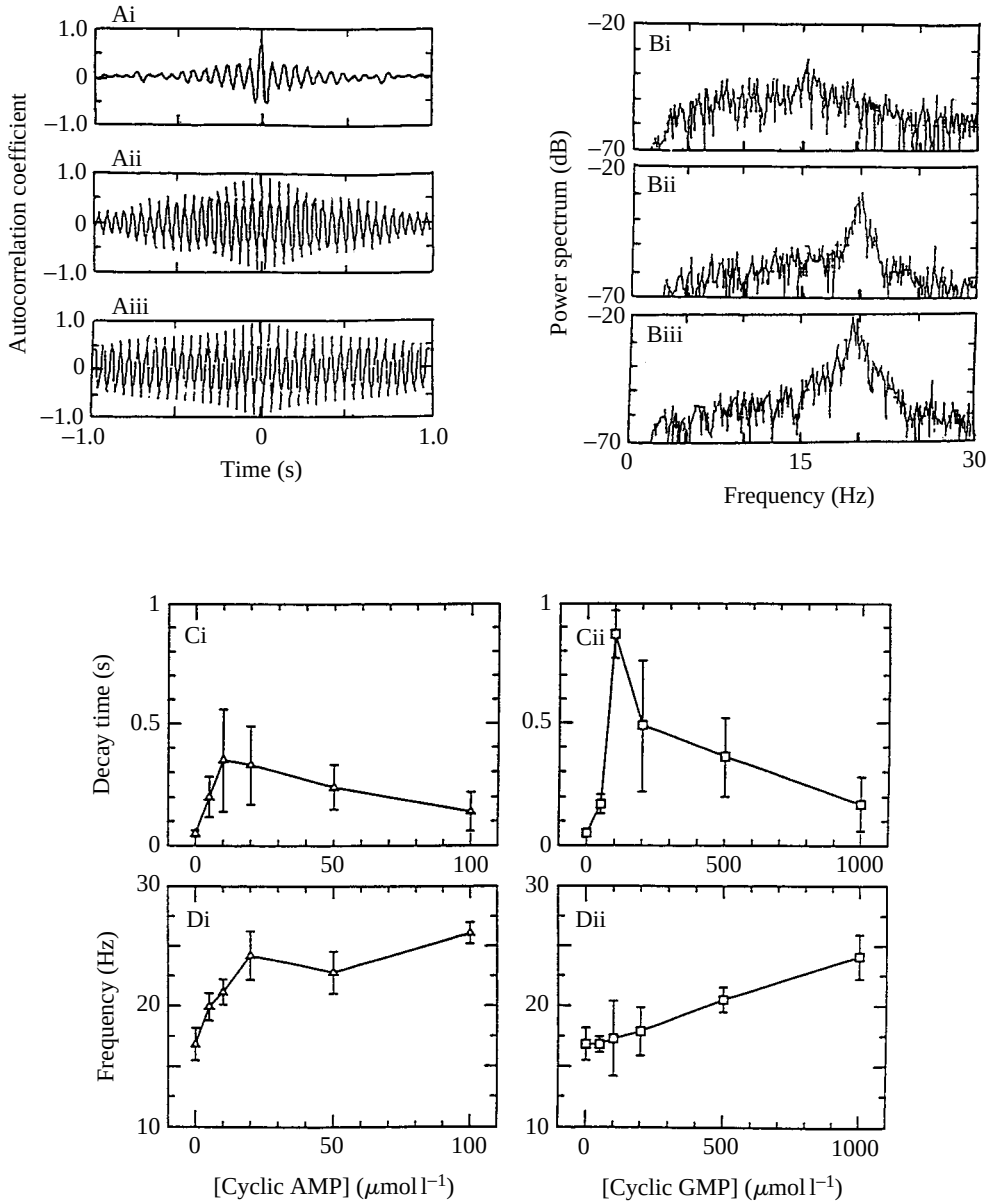

Fig. 2. Effects of cyclic AMP and cyclic GMP on the autocorrelation coefficients and the power spectra of ciliary fields. The photocurrent of the ciliary field recorded for 40–80 s after reactivation was used to calculate the autocorrelation in basic (Ai), 10 μmol l^{-1} cyclic AMP (Aii) or 100 μmol l^{-1} cyclic GMP (Aiii) reactivation media and the corresponding power spectra (Bi–iii). Decay times at different concentrations of cyclic AMP (Ci) and cyclic GMP (Cii) were obtained from the autocorrelation. Wave frequencies in different concentrations of cyclic AMP (Di) and cyclic GMP (Dii) were obtained from the power spectra. Values are means ± S.D. (N=3).

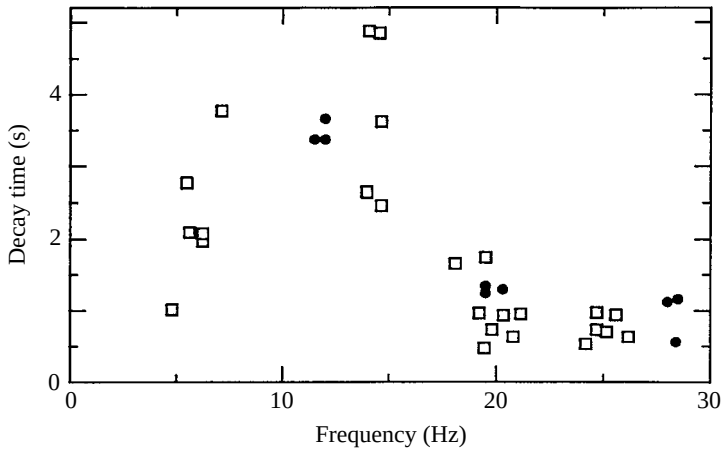

Fig. 3. Decay times at different wave frequencies. Ciliated cortical sheets were reactivated by perfusions of 100 μmol l^{-1} cyclic GMP medium containing different ATP concentrations in the range 0.2–1 mmol l^{-1}. Decay times and wave frequencies were obtained from the photocurrent recorded for 40–80 s after the reactivation. Decay times and wave frequencies were also measured from living cells held by glass microneedles. Data points were obtained from three cortical sheets (□) and three living cells (●).

in living cells. When the ATP concentration in the cyclic GMP reactivation medium was successively increased from 0.2 to 1 mmol l^{-1}, the wave frequency of cortical sheets rose gradually (Okamoto and Nakaoka, 1994). As the frequency rose, the decay time of the autocorrelation coefficient increased steeply to approximately 4 s (when the ATP concentration was approximately 1 mmol l^{-1}) then decreased gradually (Fig. 3, squares). The decay times of living cells held by glass microneedles (Fig. 3, circles) had a similar distribution.

The stabilities of the waves produced by cortical sheets in the presence of cyclic GMP were similar to those of the living cell.

Cross correlation between two points on the ciliary field

Metachronal waves are produced by coordinated interactions between neighbouring cilia arranged in the same plane. To confirm this, we calculated cross correlation coefficients between two points on the ciliary field in either the cyclic AMP or cyclic GMP reactivation medium. The cross correlation is the correlation between the waves observed simultaneously at two different points. It also indicates the time when the correlation between two points is maximal.

The cross correlation coefficient along the direction of wave propagation reached a maximum at about the time when the wave propagated from the first to the second point (Fig. 4Ai,Bi). The maximum value declined linearly with an increase in the distance between the original and second points (Fig. 4Ci). The distances for the cross correlation coefficient to decline to 0.5 were approximately 40 μm in the cyclic AMP reactivation medium and 80 μm in the cyclic GMP reactivation medium.

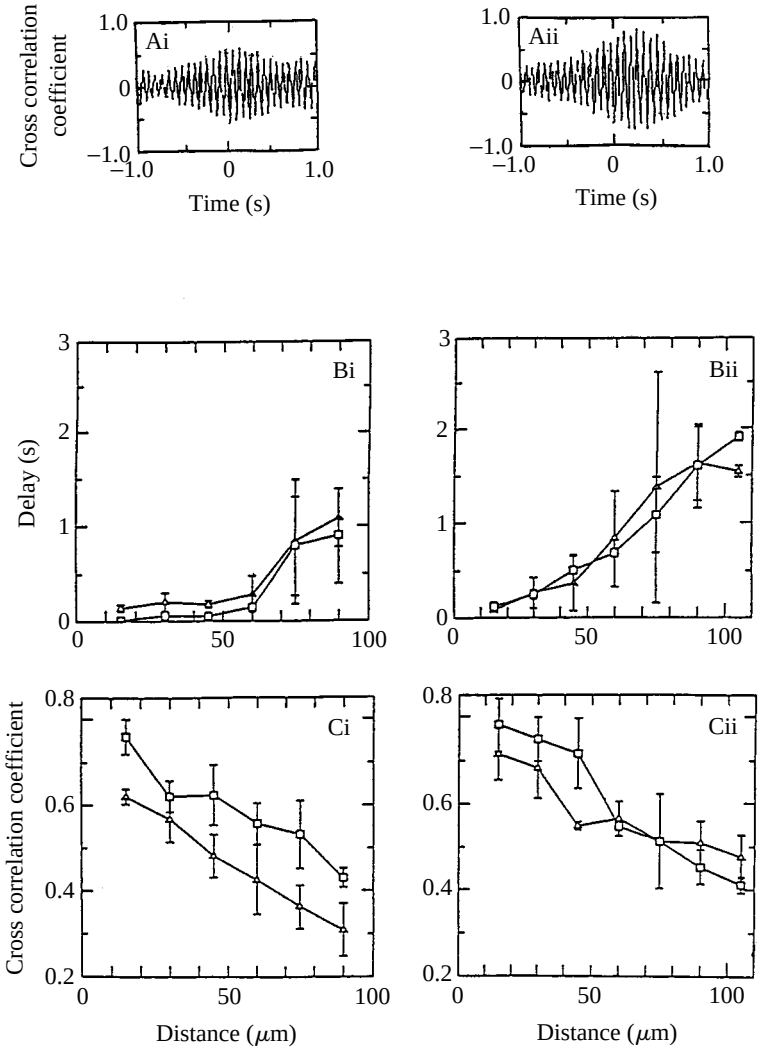

Fig. 4. Cross correlation of the reconstituted metachronal waves. The ciliated cortical sheet was reactivated by cyclic AMP or cyclic GMP medium. Cross correlation coefficients between two points 30 μm apart were calculated along the wave crest line (Ai) and the direction of wave propagation (Aii). The delay to the time when the cross correlation coefficient reached a maximum was plotted as a function of the distance along the wave crest line (Bi) and of the direction of wave propagation (Bii). The maximum value of the cross correlation coefficient was plotted as a function of the distance along the wave crest line (Ci) and of the direction of wave propagation (Cii). These measurements were made in the presence of 10 μmol l^{-1} cyclic AMP (△) or 100 μmol l^{-1} cyclic GMP (□). Values are means ± S.D. (N=3).

When the cross correlations along the crest line of the metachronal waves were calculated, the maximum value of the correlation coefficient also shifted from time zero (Fig. 4Aii). This delay time was 3–4 times longer than that along the direction of wave

propagation and increased in proportion to the distance along the crest line (Fig. 4Bii). The peak value of the correlation coefficient declined linearly with the distance (Fig. 4Cii). The reduction to 0.5 of its value occurred at approximately 80 μm in both the cyclic AMP and the cyclic GMP media.

Effect of thiophosphorylation on the metachronal wave

In order to examine whether the metachronal waves generated in the cyclic AMP or cyclic GMP reactivation media were related to protein phosphorylation, we applied ATP-γ-S in place of ATP. It has been shown that ATP-γ-S is a substrate for protein kinases and that the thiophosphorylated proteins are resistant to phosphatases (Gratecos and Fisher, 1974; Sherry *et al.* 1978).

Perfusion of ATP-γ-S medium over the ciliated cortical sheet completely stopped the ciliary movements, but following reperfusion with the basic reactivation medium, the ciliary movements restarted. When ATP-γ-S medium containing cyclic AMP was perfused first, the ciliary movements reactivated by subsequent perfusion of the basic reactivation medium showed no propagating waves (Fig. 5Ai) and the decay time of the autocorrelation coefficient was similar to that in the case of single perfusion of the basic reactivation medium (Fig. 5Bi). A subsequent perfusion with the cyclic AMP reactivation medium increased the decay time and generated metachronal waves.

In contrast, when ATP-γ-S medium containing cyclic GMP was perfused first, the subsequent perfusion of the basic ATP medium generated propagating waves (Fig. 5Aii). Although the waves were gradually weakened with time after perfusion of the basic reactivation medium, they persisted for at least 5 min following perfusion. The decay time of the autocorrelation coefficient measured approximately 1 min later was 0.4 s (Fig. 5Bii), which was shorter than that in the cyclic GMP reactivation medium.

Discussion

When ciliary movements of a cortical sheet prepared from detergent-extracted *Paramecium* cells were reactivated by an ATP reactivation medium, no propagating waves were generated without the addition of cyclic nucleotides (Fig. 1A). After perfusion of a reactivation medium containing either cyclic AMP or cyclic GMP, the ciliary field generated propagating metachronal waves (Fig. 1B,C).

Naitoh and Kaneko (1972, 1973) have previously demonstrated metachronal waves in detergent-extracted cells without the addition of cyclic nucleotides. We confirmed this and found that the detergent-extracted cells contained considerable amounts of cyclic AMP and cyclic GMP (intracellular concentrations in the micromolar range; K. Okamoto and Y. Nakaoka, unpublished data). During preparation of the ciliated cortical sheet in the present study, therefore, the remaining cyclic nucleotides were washed out, presumably causing the disappearance of the propagating metachronal waves in the basic reactivation medium. The reversible effect of cyclic nucleotides on the formation of waves by the ciliated cortical sheet supports this view.

The wave pattern of the ciliated cortical sheet in the cyclic AMP reactivation medium

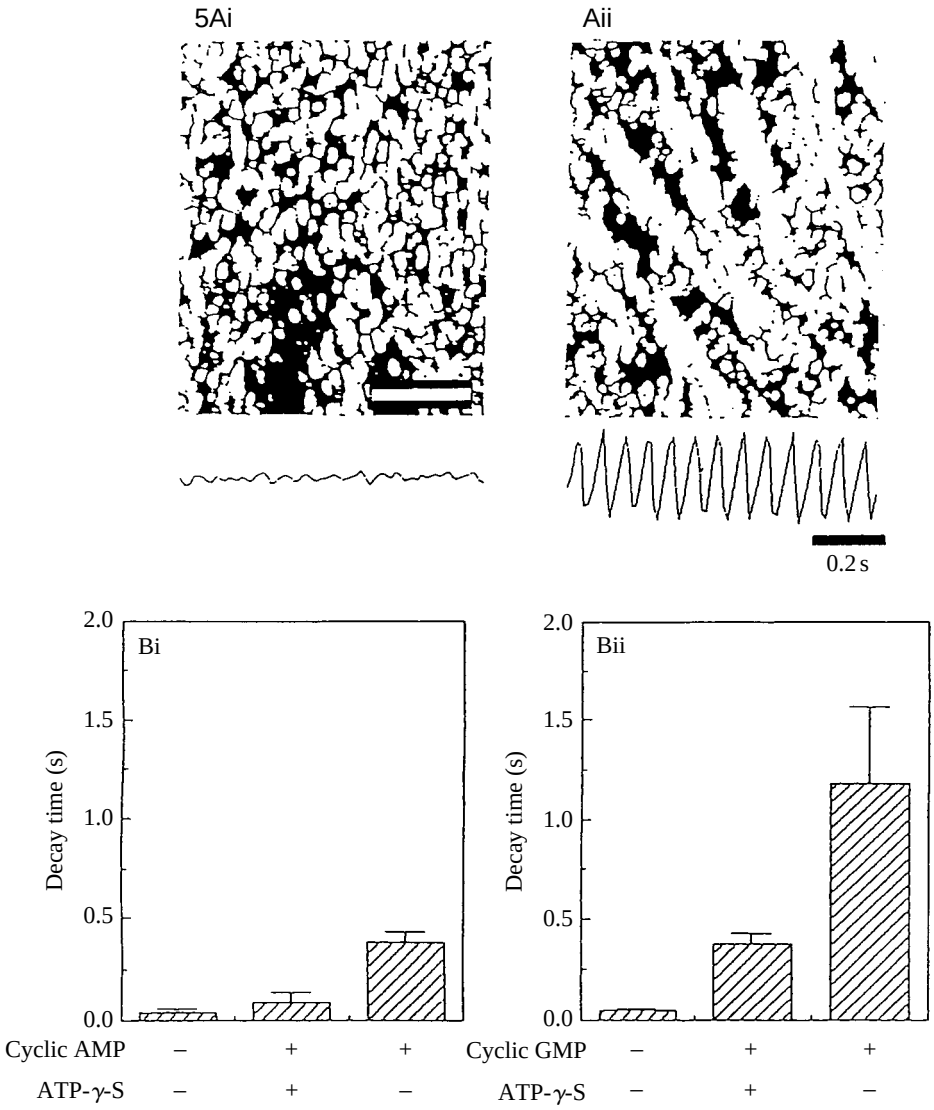

Fig. 5. Effects of preincubation in ATP-γ-S plus cyclic nucleotide medium on reactivation. For testing cyclic AMP, the ciliated cortical sheet was initially reactivated in the basic medium (−cyclic AMP, −ATP-γ-S), then ATP-γ-S medium containing 10 μmol l^{-1} cyclic AMP was perfused for 15 min. After that, the ciliated cortical sheet was reactivated in the basic ATP medium (Ai; +cyclic AMP, +ATP-γ-S). Finally, 10 μmol l^{-1} cyclic AMP reactivation medium was perfused (+cyclic AMP, −ATP-γ-S). For testing cyclic GMP, a ciliated cortical sheet was initially reactivated in the basic medium (−cyclic GMP, −ATP-γ-S), then ATP-γ-S medium containing 100 μmol l^{-1} cyclic GMP was perfused for 15 min. After that, the basic reactivation medium was perfused (Aii; +cyclic GMP, +ATP-γ-S). Finally, 100 μmol l^{-1} cyclic GMP reactivation medium was perfused (+cyclic GMP, −ATP-γ-S). Photographs were taken and the accompanying photocurrents (Ai,ii) were measured approximately 30 s after perfusion of the basic reactivation medium. Scale bar, 15 μm. Decay times (Bi,ii) were determined from the photocurrent measured between 40 and 80 s after perfusion of the respective reactivation medium. Values are mean + S.D. (N=10).

presumably corresponds to forward swimming with right-hand spiralling. Right-hand spiral swimming of detergent-extracted cells in the presence of cyclic AMP has previously been reported (Bonini and Nelson, 1988). In contrast, both the wavelength and the direction of wave propagation in the cyclic GMP reactivation medium correspond to that of the living cell swimming forwards with left spiralling (Parducz, 1967; Machemer, 1974). Such a correspondence suggests that cyclic GMP is involved in the formation of natural metachronal waves.

The maximum value of the decay time of the autocorrelation coefficient in the cyclic GMP reactivation medium was approximately three times longer than that in the cyclic AMP reactivation medium (Fig. 2Ci,ii) and was similar to that of the living cell (Fig. 3). The cross correlation coefficients in the cyclic GMP reactivation medium were mostly larger than those in the cyclic AMP reactivation medium (Fig. 4Ci,ii), showing that the metachronal waves formed in the cyclic GMP medium were more stable than those in the cyclic AMP medium.

The experiments using ATP-γ-S suggested the possibility that some phosphorylations stimulated by cyclic GMP are related to the generation of waves. In the case of cyclic AMP, however, there is no evidence suggesting that cyclic-AMP-dependent phosphorylation is involved in the generation of waves, although thiophosphorylation stimulated with cyclic AMP has been demonstrated in *Paramecium* (Nakaoka and Ooi, 1985; Hamasaki *et al*. 1991). Cyclic AMP and cyclic GMP appear to generate waves in different ways.

Measurements of the brightness fluctuation in the ciliary field and calculations of the correlation coefficient revealed some characteristics of the metachronal waves. First, cyclical changes in the brightness of the ciliary field usually accompanied beats of lower frequencies (Fig. 1B,C). Such beats seem to originate from coordinated interactions between cilia. Second, the stabilities of the waves were markedly different under the different conditions that gave rise to propagating waves. The decay times of the autocorrelation coefficient were distributed between 0.2 and 5 s (Figs 2Ci,ii, 3). The ciliary beating frequencies were approximately 20 Hz, so the coefficient decreases to half its value after 4–80 wave cycles. Third, the decay time showed peaks at appropriate concentrations of cyclic AMP, cyclic GMP and ATP (Figs 2Ci,ii, 3). These agents affected both the wave frequency and the ciliary coordination in a concentration-dependent manner.

Cross correlation coefficients between two points on the ciliary field showed some interesting characteristics. For example, the maximum cross correlation coefficient along the wave crest occurred after a delay that was 3–4 times longer than that along the direction of wave propagation (Fig. 4Bi,ii). This delay corresponds to the time taken for the signal underlying ciliary interactions to propagate along a defined distance. The wave crest was almost parallel to the direction of the effective stroke and the wave propagated almost parallel to the ciliary arrangements, which showed successive phases in the recovery stroke (Parducz, 1967; Machemer, 1974). Because the ciliary motion of the effective stroke is much faster than that of the recovery stroke, the signal should propagate along the wave crest more rapidly than in the direction of wave propagation. This expectation is opposite to the present results. The fast signal propagation that accompanied ciliary

motion was, instead, found in the direction of wave propagation, suggesting that the ciliary interaction underlying wave formation occurs mainly during the recovery stroke.

Another interesting characteristic is that the distance over which the cross correlation coefficients decreased to 0.5 was approximately 80 μm in the cyclic GMP reactivation medium (Fig. 4Ci,ii). Under the same conditions, the time over which the autocorrelation coefficients measured at one point decayed to 0.5 was 0.9 s (Fig. 2Cii). The wave frequency was approximately 20 Hz and the wavelength was approximately 10 μm, so the cross correlation should decrease to 0.5 over 180 μm. This contradiction between auto correlation and cross correlation suggests that the wave stability decreases as the wave propagates across the surface.

The ciliated cortical sheet of *Paramecium* is suitable for the analysis of metachronal waves. Using such sheets, we have attempted to correlate the metachronal waves to the motion of individual cilia (Okamoto and Nakaoka, 1994).

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