Control of the Ciliary Beat by Cyclic Nucleotides in Intact Cortical Sheets from Paramecium

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Control of the Ciliary Beat by Cyclic Nucleotides in Intact Cortical Sheets from *Paramecium*

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ABSTRACT—The locomotor behavior of *Paramecium* depends on the ciliary beat direction and beat frequency. Changes in the ciliary beat are controlled by a signal transduction mechanism that follows changes in the membrane potential. These events take place in cilia covered with a ciliary membrane. To determine the effects of second messengers in the cilia, cortical sheets were used with intact ciliary membrane as a half-closed system in which each cilium is covered with a ciliary membrane with an opening to the cell body. Cyclic nucleotides and their derivatives applied from an opening to the cell body affected the ciliary beat. cAMP and 8-Br-cAMP increased the beat frequency and the efficiency of propulsion and acted antagonistically to the action of Ca^{2+}. cGMP and 8-Br-cGMP increased the efficiency of propulsion accompanying clear metachronal waves but decreased the beat frequency. These results indicate that the cyclic nucleotides affect target proteins in the ciliary axonemes surrounded by the ciliary membrane without a membrane potential and increase the efficiency of propulsion of the ciliary beat. In vitro phosphorylation of isolated ciliary axonemes in the presence of cyclic nucleotides and their derivatives revealed that the action of cAMP was correlated with the phosphorylation of 29-kDa and 65-kDa proteins and that the action of cGMP was correlated with the phosphorylation of a 42-kDa protein.

Key words: flagella, dynein, signal transduction, phosphorylation, cAMP

INTRODUCTION

The locomotor behavior of *Paramecium* depends on the ciliary beat direction and beat frequency. Changes in the beat direction and beat frequency are correlated with changes in the membrane potential (Machemer, 1988a, 1988b). External stimuli result in membrane permeability to ions, which in turn results in changes in the membrane potential. Together with the changes in membrane potential, the intraciliary concentrations of second messengers change. Membrane depolarization elicits a Ca^{2+} action potential, which causes an increase in the intraciliary Ca^{2+} concentration, resulting in ciliary reversal and transient backward swimming (Eckert, 1972; Naitoh and Kaneko, 1972). The increase in intraciliary Ca^{2+} concentration also increases the intracellular cGMP concentration (Schultz *et al.*, 1986). On the other hand, membrane hyperpolarization increases the internal cAMP accompanied by ciliary augmentation, which results in transient faster forward swimming (Bonini *et al.*, 1986). This relationship among membrane potentials, intracellular second messenger levels, and

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ciliary responses, however, is not necessarily consistent with the results of voltage-clamp experiments, in which cAMP injected into a *Paramecium* cell body did not increase the ciliary beat frequency (Nakaoka and Machemer, 1990). To make clear the signal transduction mechanism that controls ciliary movement, further studies have been required.

The above-mentioned signal transduction takes place in cilia covered with ciliary membranes. Cyclic nucleotides are presumably produced by enzymes that reside in the ciliary membrane (Schultz and Klumpp, 1984; Schultz *et al.*, 1987; Gustin and Nelson, 1987). Schultz *et al.* (1992) have suggested that an adenylyl cyclase is itself a K^+ channel present in the ciliary membrane and that the hyperpolarization-activated K^+ efflux directly regulates this enzyme. Cyclic nucleotides produced by these enzymes activate some protein kinases (Mason and Nelson, 1989a, b; Hochstrasser and Nelson, 1989), and the kinases phosphorylate some axonemal proteins (Hamasaki *et al.*, 1989; Bonini and Nelson, 1990; Noguchi *et al.*, 2000). Not only phosphorylation but also dephosphorylation is thought to regulate the sliding between dyneins and outer doublet microtubules in ciliary axonemes (Noguchi *et al.*, 2003).

These findings have been obtained using various experimental conditions, for example, in demembranated

cell models and in voltage-clamped cells (Pech, 1995). The demembranation with detergents, however, may damage the signal transduction pathways. In this study, cortical sheets with an intact ciliary membrane were used to produce a half-closed system that consists of cilia each covered with a ciliary membrane with an opening to the cell body (Noguchi *et al.*, 2001). Using this experimental system, the effects of second messengers in the ciliary axoneme covered with a ciliary membrane were tested. The results indicate that the cyclic nucleotides affect target proteins in the milieu surrounded by the ciliary membrane without a membrane potential, and increase the efficiency of propulsion of the ciliary beat. In vitro phosphorylation of isolated ciliary axonemes by cyclic nucleotides and their derivatives revealed that the action of cAMP was correlated with the phosphorylation of 29-kDa and 65-kDa proteins and that the action of cGMP may be correlated with the phosphorylation of a 42-kDa protein.

MATERIALS AND METHODS

Preparation of ciliated cortical sheets from live cells

Paramecium caudatum (stock G3) was cultured in a hay infusion. Cells were grown to the late-logarithmic phase at 25°C. The preparation of ciliated cortical sheets from live cells (intact cortical sheets) and the reactivation of cilia were essentially the same as in our previous paper (Noguchi *et al.*, 2001). The cortical sheets were successively perfused with reactivation solutions. All the reactivation solutions contained 2 mM MgCl$_2$, 50 mM potassium acetate, 0.4 mM phosphoarginine and 10 mM Tris-maleate buffer (pH 7.0) as well as ATP and the component(s) noted in the results. The free Ca^{2+} concentration in the reactivation solution was controlled by a Ca-EGTA buffer (Portzehl *et al.*, 1964) with 1 mM EGTA. Reactivation of motility in the model was carried out at 22–25°C. The movements of the cilia were observed under a dark-field microscope equipped with a 100 W mercury light source, a heat filter and a green filter, and recorded on videotape using a National WV-1550 TV camera.

Determination of the direction of fluid flow over the surface of cortical sheets

To observe the fluid flow over the ciliated cortical sheets, the sheets were perfused with reactivation solutions containing polystyrene microspheres (diameter=1 μm; Polysciences, Inc. Warrington, PA). The flow of the beads was recorded on videotapes through a National WV-1550 TV camera.

Beat frequency of the reactivated cilia

The beat frequencies of reactivated cilia were measured according to Noguchi *et al.* (2001). The frequencies of change in light intensity due to the cyclic beatings of cilia were monitored through a small hole placed above an eyepiece of a dark-field microscope. Cyclic changes in light intensity transmitted to a phototransistor through the small hole was amplified with operational amplifiers. The beat frequency was obtained by measuring the peak frequency in the fast Fourier transform (FFT) spectra (Noguchi *et al.*, 2001). The beat frequencies of the cilia on the left-hand field of cortical sheets (Noguchi *et al.*, 1991) were measured.

Isolation of cilia

Collected cells were washed three times with a washing solution (2 mM KCl, 2 mM CaCl$_2$, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris-maleate, pH 7.0). Cells were deciliated by dibucaine treatment, following Mogami and Takahashi (1983) with slight modifications (Noguchi *et al.*, 2000). Cilia were isolated from cell bodies by centrifugation repeated twice at 600g for 5 min. The pellets were discarded. The supernatant was centrifuged at 7,700 g for 10 min. The pellet was resuspended in a TMKE solution (10 mM Tris-maleate, pH 7.0, 5 mM MgCl$_2$, 20 mM potassium acetate, 1 mM EGTA) containing 0.3 mM PMSF and centrifuged. The pellet was rewashed with the TMKE solution. Isolation of cilia was monitored by dark-field microscopy. These cilia were then treated with a demembranation solution containing 0.1% Triton X-100 in TMKE solution for 10 min at 0°C. The suspension was centrifuged to pellet the axonemes. The Triton was removed from axonemes by washing twice with a TMKE solution. The pellet of axonemes was suspended in a small amount of the TMKE solution.

Phosphorylation of the axonemes

In vitro phosphorylation of the axonemes was performed following the method of Hamasaki *et al.* (1989) with slight modifications (Noguchi *et al.*, 2000). The reaction mixture contained 0.15 mg axonemes in 80 μl TMKE solution as well as test substances.

Fig. 1. Reactivation of cilia on the intact cortical sheet. A reactivated intact cortical sheet under a dark-field microscope. An intact cortical sheet was reactivated with 0.05 mM Mg-ATP, 50 μM cGMP, 1 mM EGTA, 10 mM Tris-maleate buffer (pH 7.0), and 0.4 mM phosphoarginine under a dark-field microscope. The frame of the video recording is converted to a view from the outside of the cell. The top of the frame is the anterior part of the cell. Reactivated cilia beat accompanying a metachronal wave (indicated by arrowheads). A bead indicated in the circle flows toward the posterior region. The asterisk indicates the oral-groove region. Bar, 100 μm. (Moving image can be seen at http://www.sci.toyama-u.ac.jp/~noguchi/zs04/zs04.html)

Phosphorylation by endogenous protein kinases was started by the addition of 20 μl of γ-[^{32}P]ATP to reach the final concentration of 1 μM ATP. The ATP concentration of the γ-[^{32}P]ATP was 5 μM, and the radioactivity was adjusted to 10 or 20 μCi with adenosine 5'-γ-[^{32}P]triphosphate (specific activity 6000 Ci/mmol from Du Pont-New England Nuclear). Immediately after 10 min incubation at 0°C, the reaction mixture was centrifuged at 10,000g for 10 min. The pellet was directly suspended in an SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.5% bromophenol blue, 62.5 mM Tris-HCl, pH 6.8) and incubated at 100°C for 2 min. These SDS-treated samples were then subjected to SDS-PAGE or stored at −20°C for further use. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis and autoradiography

SDS-PAGE was performed following a modification of the procedure of Laemmli (1970) using a 3–15% linear gradient acrylamide gel containing a 0–19% glycerol gradient run on a 20×16×0.1 cm slab gel. The gels were stained with Coomassie Blue R-250 for 15 min or with silver (Blum *et al.*, 1987) and dried on filter paper. Molecular weight standards were obtained from Bio-Rad.

To produce the autoradiograms, Fuji medical X-ray film RX (Fuji, Kanagawa) with an intensifying screen (Kasei Optonix, Tokyo) was placed over the dried gels for 1 to a few days and developed.

RESULTS

The direction of fluid flow over the surface of reactivated cortical sheets

The reactivated cilia on the cortical sheet caused fluid flows depending on the ciliary beat direction (Fig. 1). The direction of the fluid flow over the reactivated cortical sheets in response to Ca^{2+} concentration corresponded basically to the ciliary orientation of the cortical sheet from Triton-glycerol-extracted *Paramecium* (Noguchi *et al.*, 1991). In the presence of micromolar Ca^{2+}, the direction of the fluid flow

was toward the anterior of the cell that corresponded to the ciliary reversal (Fig. 3A, E). In the absence of Ca^{2+}, the fluid on the oral groove region flowed in the posterior direction that corresponded to the ciliary power stroke direction of forward swimming. However, the fluid on the both sides of the oral groove flowed into the oral groove region (Fig. 2A, E). The addition of cAMP or cGMP to the reactivation solutions without Ca^{2+} induced a uniform fluid flow toward the posterior region all over the cortical surface (Fig. 2D, H). The concentrations of cAMP and cGMP that were enough to induce such a flow were 10 and 1 μM, respectively (Fig. 2D, H). In the presence of cGMP, a clear metachronal wave was observed (Fig. 1).

In the presence of 1.2 μM Ca^{2+}, the direction of fluid flow was toward the anterior that corresponded to the ciliary reversal (Fig. 3A, E). The addition of 10 μM cAMP to the reactivation solution in the presence of 1.2 μM Ca^{2+} induced fluid flow toward the posterior end of the cell on the oral-groove region (Fig. 3C). Further addition of cAMP up to 25 μM induced fluid flow in the posterior direction all over the cortical surface (Fig. 3D). Over 50 μM cGMP induced only a slight change in fluid flow, but it could not change direction of the fluid flow from the anterior to the posterior direction (Fig. 3).

Effects of cyclic nucleotide derivatives on the fluid flow over the surface of reactivated cortical sheets

It is known that high concentration cGMP activates cAMP-dependent protein kinase (A-kinase) but 8-Br-cGMP is a poor activator of *Paramecium* type II A-kinase (Mason and Nelson, 1989b). To clarify the difference between cAMP and cGMP in the effect on propulsive force of ciliary beat,
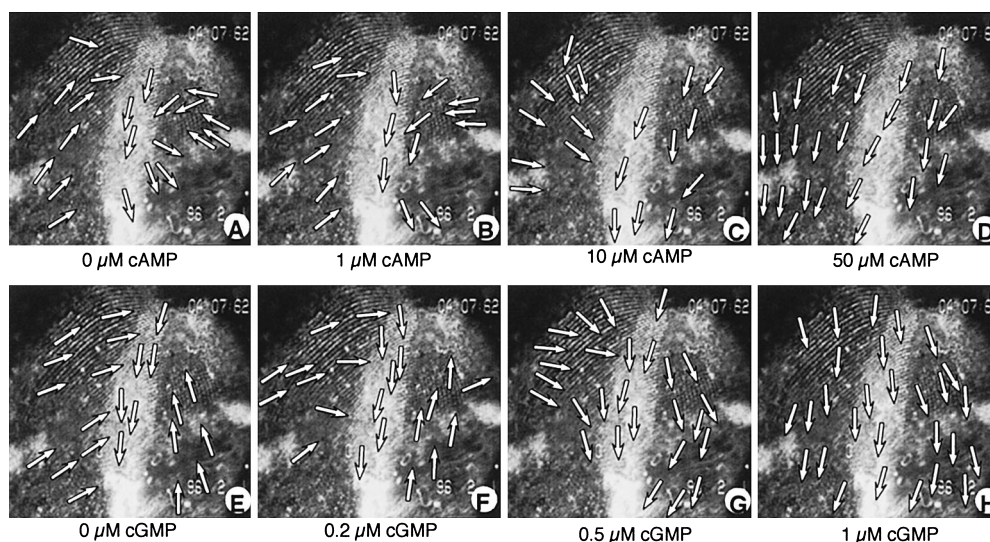

| 0 μM cAMP | 1 μM cAMP | 10 μM cAMP | 50 μM cAMP |

| 0 μM cGMP | 0.2 μM cGMP | 0.5 μM cGMP | 1 μM cGMP |

Fig. 2. Direction of fluid flow over the surface of reactivated cortical sheets in the absence of Ca^{2+}. The direction of fluid flow is expressed diagrammatically by arrows on typical cortical-sheet images. Usually, the fluid flow and cortical surface cannot be observed simultaneously because the focal plane of the flowing plastic beads is different from that of the cortical surface. The cortical sheets were perfused successively with reactivation solutions containing 2 mM MgCl$_2$, 0.5 mM ATP, 0.4 mM phosphoarginine, 50 mM potassium acetate, 1 mM EGTA, 10 mM Tris-maleate buffer (pH 7.0) and cAMP (A–D) or cGMP (E–H). The frame is a view from inside the cell. The top of the frame is the anterior direction of the cell. Bar, 10 μm.
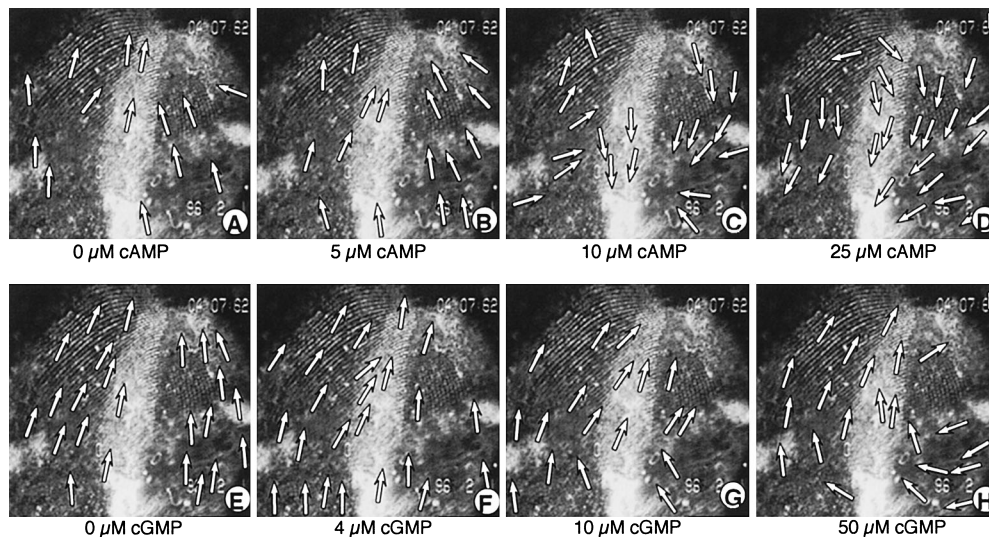
Fig. 3. Direction of fluid flow over the surface of reactivated cortical sheets in the presence of 1.2 μM Ca^{2+}. The direction of fluid flow is expressed diagrammatically by arrows on typical cortical-sheet images. The cortical sheets were perfused successively with reactivation solutions containing 2 mM MgCl$_2$, 0.5 mM ATP, 0.4 mM phosphoarginine, 50 mM potassium acetate, 1.2 μM Ca^{2+}, 10 mM Tris-maleate buffer (pH 7.0), and cAMP (A–D) or cGMP (E–H). The frame is a view from inside the cell. The top of the frame is the anterior direction of the cell. Bar, 10 μm.

Table 1. Effects of cyclic nucleotides on the ciliary beat of the intact cortical sheet from *Paramecium*

Cyclic nucleotides	Concentration required for inducing uniform fluid flow (μM)	Concentration required for overcoming the ciliary reversal (μM)
cAMP	> 5	> 25
8-Br-cAMP	> 0.5	> 25
N^6-monobutyryl cAMP	> 5	–
2'-deoxy cAMP	–	–
2'-O-monobutyryl cAMP	–	–
N^6,2'-O-dibutyryl cAMP	–	–
cGMP	> 0.5	–
8-Br-cGMP	> 0.5	–
N^2-monobutyryl cGMP	> 0.5	–
N^2,2'-O-dibutyryl cGMP	–	–

The effectiveness of cyclic nucleotides in the absence of Ca^{2+} was expressed as minimal concentrations that were sufficient to induce a uniform flow in the posterior direction all over the cortical surface. The effectiveness of cyclic nucleotides in the presence of 1.2 μM Ca^{2+} was expressed as minimal concentrations that were sufficient to overcome a Ca^{2+}-induced ciliary reversal. –, no effect at 100 μM.

the effects of cyclic nucleotide derivatives were tested. Among the tested cAMP derivatives, 8-Br-cAMP and N^6-monobutyryl cAMP induced a uniform fluid flow on the cortical sheets toward the posterior end of the cell. The concentration of 8-Br-cAMP that was sufficient to induce uniform flow toward the posterior end was as low as that of cGMP (0.5 μM) (Table 1). Among the tested cGMP derivatives, 8-Br-cGMP and N^2-monobutyryl cGMP were as effective as cGMP to induce uniform fluid flow toward the posterior end of the cell. The concentrations of 8-Br-cGMP and N^2-monobutyryl cGMP that were sufficient to induce uniform fluid flow in the posterior direction were as low as that of cGMP (0.5 μM) (Table 1). In the presence of cGMP and 8-

Br-cGMP, a clear metachronal wave was observed. Among the tested cAMP derivatives, only 8-Br-cAMP changed the fluid flow from the anterior to the posterior in the reactivation solution containing 1.2 μM Ca^{2+}. No cGMP derivatives effectively caused fluid flow toward the posterior region in the presence of 1.2 μM Ca^{2+} (Table 1).

Changes in beat frequency induced by cyclic nucleotides and their derivatives

Changes in beat frequency by the addition of cAMP, cGMP, and some of their derivatives were measured to determine whether the uniform anterior-to-posterior fluid flow, induced by the addition of cyclic nucleotides, was due

to the increase in the beat frequency of cilia. As shown in Fig. 4A, cAMP and 8-Br-cAMP increased the beat frequency. Other cAMP derivatives did not increase the beat frequency. On the contrary, cGMP and 8-Br-cGMP significantly decreased the beat frequency (Fig. 4B). Other cGMP derivatives did not significantly change the beat frequency.
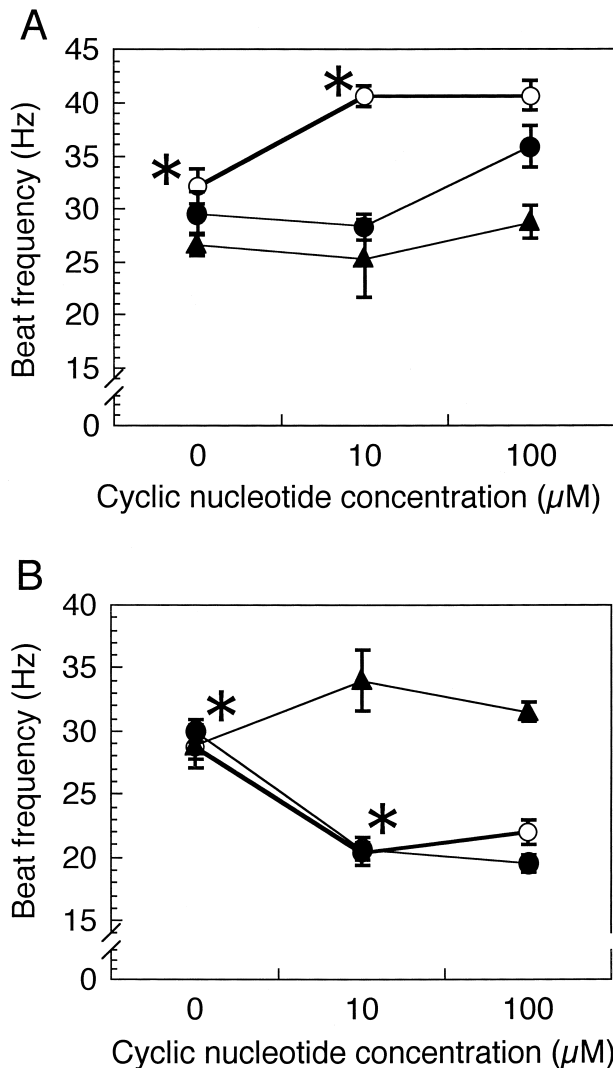

Fig. 4. Effects of cAMP, cGMP and derivatives on the beat frequency of reactivated cilia. Ciliated sheets were perfused successively with reactivation solutions containing 2 mM $MgCl_2$, 0.2 mM ATP, 0.4 mM phosphoarginine, 50 mM potassium acetate, 1 mM EGTA, 10 mM Tris-maleate buffer (pH 7.0) and cAMP or derivatives. Data from separate several preparations show means±s.e.m. **A:** ○; cAMP, ●; 8-Br-cAMP, ▲; N^6-monobutyryl cAMP. The difference in the beat frequency in the absence and in the presence of 10 μM cAMP (indicated by asterisks) was significant (*t* test: 0 μM cAMP vs. 10 μM cAMP, $p<0.01$, n=9). **B:** ○; cGMP, ●; 8-Br-cGMP, ▲; N^2-monobutyryl cGMP. The difference in the beat frequency in the absence and in the presence of 10 μM cGMP or 10 μM 8-Br-cGMP (indicated by asterisks) was significant (*t* test: 0 μM cGMP vs. 10 μM cGMP or 10 μM 8-Br-cGMP, $p<0.01$, n=9).

Phosphorylation of the axonemal proteins depending on the cyclic nucleotides and their derivatives

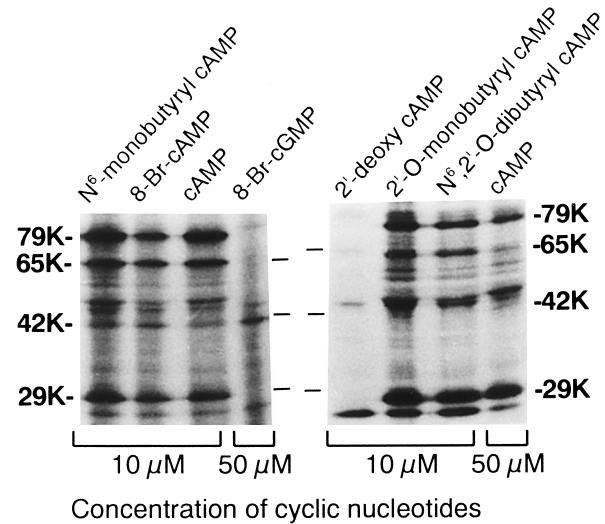To clarify the relations between the effect of the cyclic

Concentration of cyclic nucleotides

Fig. 5. Phosphorylation of axonemal proteins in the presence of cAMP and derivatives. Phosphorylated proteins were run on a 3–15% linear gradient acrylamide gel. Lanes are autoradiograms of the SDS-PAGE. In vitro phosphorylation of axonemes by endogenous kinases was performed in the presence of cAMP and derivatives. 79K, 65K, 42K, and 29K indicate cAMP-dependent phosphorylated 79-kDa, 65-kDa, 42-kDa and 29-kDa proteins in the autoradiograms, respectively. The band pattern of lane 5 is almost the same as that of the one without cyclic nucleotides. Markers between lanes 4 and 5 indicate (from top) 66, 45, and 31 kDa.
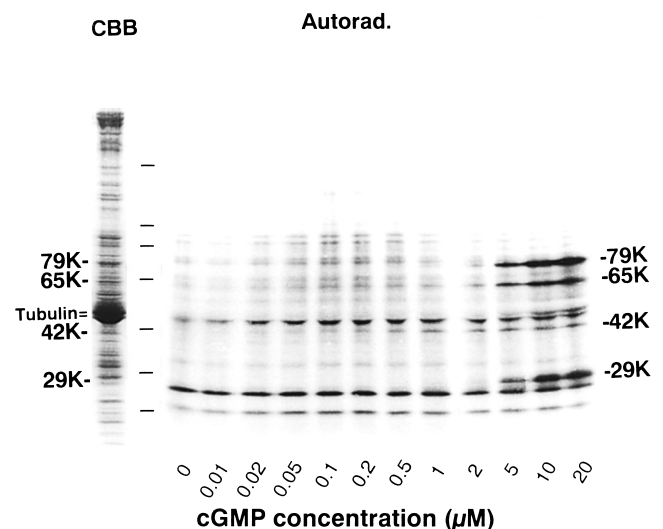

cGMP concentration (μM)

Fig. 6. Phosphorylation of axonemal proteins depending on the concentration of cGMP. In vitro phosphorylation of axonemes by endogenous kinases was performed in the presence of cGMP. The concentrations of cGMP are indicated below the lanes. Phosphorylated proteins were run on a 3–15% linear gradient acrylamide gel. CBB: band pattern stained with Coomassie Blue R 250. Autorad: autoradiogram. 79K, 65K, 42K and 29K indicate cAMP-dependent phosphorylatable 79-kDa, 65-kDa, 42-kDa and 29-kDa proteins in the autoradiograms, respectively. Markers between CBB and Autorad indicate (from top) 200, 116, 97, 66, 45, 31, and 21.5 kDa.

nucleotides and their derivatives on the ciliary activities and the phosphorylation of the axonemal proteins, in vitro phosphorylation by endogenous protein kinases was performed in the presence of cyclic nucleotides and their derivatives. Endogenous protein kinases phosphorylated 29-kDa, 65-kDa, and 79-kDa proteins in the presence of cAMP, as previously reported (Noguchi *et al.*, 2000, 2003). 8-Br-cAMP and N[6]-monobutyryl cAMP, which effectively induced uniform fluid flow in the posterior direction, stimulated the phos-

phorylation of these proteins (Fig. 5). Among ineffective cAMP derivatives in the activation of ciliary beat, 2'-O-monobutyryl cAMP and N[6],2'-O-dibutyryl cAMP stimulated the phosphorylation of the 29-kDa, 65-kDa, and 79-kDa proteins at higher concentrations (more than 2 μM cAMP) (Fig. 5). Greater than 2 μM cAMP, 8-Br-cAMP, and N[6]-monobutyryl cAMP also stimulated phosphorylation of a 42-kDa protein, in addition to the 29-kDa, 65-kDa, and 79-kDa proteins (Fig. 5). The 79-kDa phosphoprotein was specifically found in the cilia from *P. caudatum* but not in the cilia from *P. tetraurelia* and *P. multimicronucleatum* (unpublished data), and was never dephosphorylated by protein phosphatase 2C (Noguchi *et al.*, 2003).

In the presence of 0.5 μM cGMP, the 42-kDa protein was phosphorylated. Greater than 5 μM cGMP stimulated the phosphorylation of the 29-kDa, 65-kDa, and 79-kDa proteins that were usually phosphorylated in the presence of cAMP (Fig. 6). Among the cGMP derivatives, 8-Br-cGMP and N[2]-monobutyryl cGMP, which were as effective as cGMP to induce uniform anterior-to-posterior fluid flow, stimulated the phosphorylation of the 42-kDa protein (Fig. 7). 8-Br-cGMP never stimulated the phosphorylation of the 29-kDa, 65-kDa and 79-kDa proteins (Fig. 7, Table 2).

DISCUSSION

In this study, the effect of second messengers in the ciliary axoneme of *Paramecium* covered with a ciliary membrane was examined using intact cortical sheets. Cyclic nucleotides and their derivatives applied from an opening to the cell body were found to induce changes in the ciliary beat. This indicates that the cyclic nucleotides can diffuse into the ciliary matrix from a cell body through a ciliary basal opening. The reactivated ciliary beat caused fluid flow on the intact cortical sheet (Table 1; Figs. 1, 2, and 3).
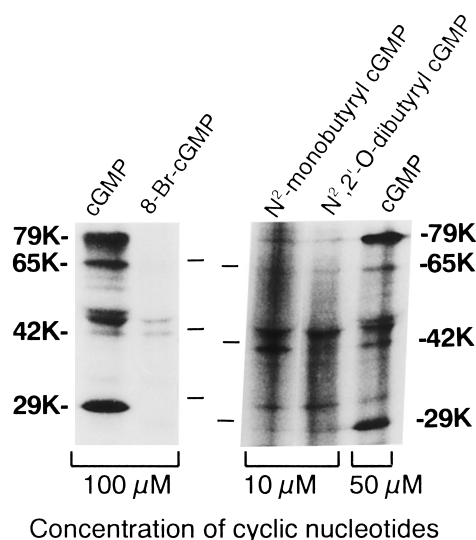

Fig. 7. Phosphorylation of axonemal proteins in the presence of cGMP and derivatives. Phosphorylated proteins were run on a 3-15% linear gradient acrylamide gel. Lanes are autoradiograms of the SDS-PAGE. In vitro phosphorylation of axonemes by endogenous kinases was performed in the presence of cGMP and derivatives. 79K, 65K, 42K, and 29K indicate cAMP-dependent phosphorylated 79-kDa, 65-kDa, 42-kDa and 29-kDa proteins in the autoradiograms, respectively. Markers between lanes 2 and 3 indicate (from top) 66, 45, and 31 kDa.

Table 2. Effects of cyclic nucleotides on the in vitro phosphorylation of axonemal proteins

Cyclic nucleotides	Concentration required for the phosphorylation of 29 and 65-kDa proteins (μM)	Concentration required for the phosphorylation of 42-kDa protein (μM)
cAMP	> 0.2	> 2
8-Br-cAMP	> 1	> 2
N[6]-monobutyryl cAMP	> 0.2	> 2
2'-deoxy cAMP	–	–
2'-O-monobutyryl cAMP	> 2	–
N[6],2'-O-dibutyryl cAMP	> 2	–
cGMP	> 5	> 0.5
8-Br-cGMP	–	> 0.5
N[2]-monobutyryl cGMP	–	> 0.5
N[2],2'-O-dibutyryl cGMP	–	–

The effectiveness of cyclic nucleotides on the in vitro phosphorylation was expressed as minimal concentrations that were sufficient to induce the phosphorylation of axonemal proteins. –, no phosphorylation was observed in the presence of 100 μM cyclic nucleotide.

In the absence of Ca^{2+}, the direction of the fluid flow was not uniform all over the cortical surface. The fluid on the oral groove region flowed toward the posterior portion, but the fluid on both sides of the oral groove flowed into oral-groove region (Fig. 2A, F). This indicates that the cilia of the oral-groove region have a stronger power stroke than those of other region. This may be effective to gather nutrients (bacteria) into a food vacuole. To cause uniform fluid flow in the posterior direction all over the cortical surface, addition of cAMP or cGMP was necessary (Fig. 2D, H). The uniform fluid flow induced by cAMP and cGMP corresponds to the fast forward swimming induced by cAMP and cGMP in the Triton-extracted *Paramecium* (Nakaoka and Ooi, 1985; Bonini and Nelson, 1988). Especially, cGMP induced a uniform flow in the posterior direction at a concentration as low as 1 μM (Fig. 2H). In addition, cGMP induced a clear metachronal wave (Fig. 1). Okamoto and Nakaoka (1994a, b) reported that cGMP stabilized the metachronal wave on the reactivated cortical sheet from a Triton-extracted model of *Paramecium multimicronucleatum*. These facts suggest that a basic level of intracellular cGMP (or cAMP) is required for the normal ciliary beat for forward swimming of *Paramecium*.

In the presence of 1.2 μM Ca^{2+}, the direction of fluid flow was posterior-to-anterior, which corresponds to ciliary reversal (Fig. 3A, E). Cyclic AMP at concentrations above 25 μM overcame the Ca^{2+} effect and restored normal ciliary beating (Fig. 3D). Cyclic AMP may regulate the orientation of the ciliary power stroke, as inferred from the studies using voltage-clamped cells (Nakaoka and Machemer, 1990), cell models (Nakaoka and Ooi, 1985; Bonini *et al.*, 1986; Bonini and Nelson, 1988), and cortical sheets (Noguchi *et al.*, 1991). The present result indicates that the cilia on the intact cortical sheet also respond to cAMP by setting the orientation of the power stroke antagonistically to the Ca^{2+} effect.

The beat frequency of reactivated cilia was increased by cAMP and 8-Br-cAMP applied from the opening to the cell body (Fig. 4A). This result does not agree with the result obtained with voltage-clamped cells injected with cAMP; they never increased ciliary beat frequency (Nakaoka and Machemer, 1990). The result obtained with intact cortical sheets indicates that the cAMP diffusing into the ciliary matrix increases the ciliary beat frequency through the function of some signal transduction pathway. In the intact cortical sheet, each ciliary axoneme is surrounded by an intact ciliary membrane without being subjected to membrane potentials or currents. In the voltage clamp conditions, on the other hand, there are unusual currents across the membrane. Such a difference in the experimental conditions may cause the difference in the effect of cAMP on the ciliary beat frequency.

The uniform fluid flow induced by the addition of cyclic nucleotides may be due to the increase in beat frequency or to the increased propulsion efficiency. The uniform fluid flow induced by cAMP and 8-Br-cAMP seems to indicate that the elevated beat frequency caused the uniform fluid flow and

increase in swimming velocity (Bonini and Nelson, 1988) . However, the uniform fluid flow induced by cGMP and 8-Br-cGMP did not support this idea, since beat frequency was significantly decreased by these cyclic nucleotides (Fig. 4B). In addition to the decrease in beat frequency, cGMP caused clear metachronal waves in the cortical sheet. This must be due to the production of an efficient effective stroke-recovery stroke cycle (Okamoto and Nakaoka, 1994b). The activated effective stroke must induce powerful propulsion and increase the swimming velocity (Nakaoka and Ooi, 1985; Bonini *et al.*, 1986; Bonini and Nelson, 1988). The forward swimming speed of live cells and Triton-extracted cell models (Nakaoka and Ooi, 1985; Bonini and Nelson, 1988) must be controlled by two factors, the beat frequency and efficiency of the ciliary power stroke.

The cyclic nucleotides and their derivatives effective in modulating ciliary movements were expected to stimulate the phosphorylation of some axonemal proteins. The cAMP derivatives, 8-Br-cAMP and N^6-monobutyryl cAMP, which effectively induce the uniform fluid flow toward the posterior end, stimulated the phosphorylation of the 29 -kDa and 65-kDa proteins (Fig. 5). Cyclic AMP and 8-Br-cAMP also increased the beat frequency (Fig. 4A). Therefore, in live cells, increased intraciliary cAMP produced by the voltage-dependent adenylyl cyclase (Schultz *et al.*, 1992; Weber *et al.*, 2004), seems to induce a ciliary augmentation through the phosphorylation of these proteins, especially the 29 kDa-protein, which stimulates the sliding speed of 22S dynein (Hamasaki *et al.*, 1991; Barkalow *et al.*, 1994). Some cAMP derivatives that stimulated the phosphorylation of 29-kDa and 65-kDa proteins at higher concentrations (Table 2) did not induce uniform fluid flow (Table 1). The efficiency of phosphorylation or the kinetics of the phosphoprotein turnover may be important for effective regulation of the ciliary activity.

Little is known about the molecular mechanism of the antagonistic action between cAMP and Ca^{2+}; i.e., cAMP and 8-Br-cAMP overcame the Ca^{2+}-induced ciliary reversal (Fig. 3 and Table 1). The phosphorylations of the 29-kDa and the 65-kDa proteins may be involved in this antagonism. The 65-kDa phosphoprotein is especially intimately involved in this antagonism because, upon trypsin digestion, this phosphoprotein disappears in parallel with the disappearance of the antagonism (Noguchi *et al.*, 2000). In addition, it seems to be localized to the dynein complex (Ogawa and Noguchi, 2003).

In the presence of 0.5 μM cGMP, the 42-kDa protein was phosphorylated (Fig. 6). In addition to this, higher concentrations (above 5 μM) of cGMP stimulated the phosphorylation of the 29-kDa, 65-kDa and 79-kDa proteins that were usually phosphorylated in the presence of cAMP (Fig. 6). Among the cGMP derivatives, only 8-Br-cGMP and N^2-monobutyryl cGMP, which were as effective as cGMP to induce a uniform fluid flow toward the posterior region, stimulated the phosphorylation of the 42-kDa protein (Fig. 7); the 29-kDa, 65-kDa and 79-kDa proteins were not phosphory-

lated in the presence of these derivatives. 8-Br-cGMP is known to be a poor activator of the cAMP-dependent protein kinase of *Paramecium* (Mason and Nelson, 1989b), although it potently activates the cGMP-dependent protein kinase (Miglietta and Nelson, 1988). These results indicate that 29-kDa and 65-kDa phosphoproteins are not involved in the cGMP-dependent production of the effective beat cycle that results in a metachronal wave. The 42-kDa phosphoprotein may be a key protein in the cGMP-dependent signal transduction pathway in the *Paramecium* cilia.

The differential role of cAMP and cGMP in a production of the propulsive force has been unclear. Our results suggest that cGMP produces the propulsive force of cilia by producing an efficient effective stroke-recovery stroke cycle through the phosphorylation of a 42-kDa protein. On the other hand, cAMP increases the propulsive force by increasing the beat frequency and the power of the effective stroke in the posterior direction through the phosphorylations of 29-kDa and 65-kDa proteins.

ACKNOWLEDGEMENTS

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