Differential Regulation of *Paramecium* Ciliary Motility by cAMP and cGMP

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Abstract. cAMP and cGMP had distinct effects on the regulation of ciliary motility in *Paramecium*. Using detergent-permeabilized cells reactivated to swim with MgATP, we observed effects of cyclic nucleotides and interactions with Ca^{2+} on the swimming speed and direction of reactivated cells. Both cAMP and cGMP increased forward swimming speed two- to threefold with similar half-maximal concentrations near 0.5 μM. The two cyclic nucleotides, however, had different effects in antagonism with the Ca^{2+} response of backward swimming and on the handedness of the helical swimming paths of reactivated cells. These results suggest that cAMP and cGMP differentially regulate the direction of the ciliary power stroke.

DYNAMIC regulation of cellular motility is strikingly displayed in the swimming behavior of the unicellular eukaryote *Paramecium*. This ciliated cell can rapidly modulate both beat frequency and the direction of the ciliary power stroke to swim transiently backward or forward at various speeds (Eckert et al., 1976). These behavioral responses are elicited by stimuli that change the cell membrane potential (Eckert et al., 1976) and are mediated by Ca^{2+} and cAMP. Ca^{2+} couples membrane depolarization to backward swimming (Eckert et al., 1976; Kung and Saimi, 1982; Naitoh and Kaneko, 1972), and cAMP regulates forward swimming speed by increasing upon membrane hyperpolarization (Hennessey et al., 1985; Nakaoka and Ooi, 1985; Bonini et al., 1986). Although evidence suggests that cilia and flagella may typically use Ca^{2+} and cAMP as messengers for beat modulation (Tash and Means, 1983; Satir, 1985), the role of cGMP is less clear. Cilia and flagella have guanylate cyclase (Gray and Drummond, 1976; Schultz and Klumpp, 1984) and modify motion in response to cGMP (Lindemann, 1978; Nakaoka and Ooi, 1985); however, a function of cGMP distinct from that of cAMP has not been demonstrated previously. Little is also known regarding the physiological or molecular details of integration among the various second messenger systems.

To provide a framework for more detailed molecular studies, we have analyzed the swimming behavior of permeabilized paramecia in response to cAMP and cGMP, and the interactions of the two cyclic nucleotides with Ca^{2+}. *Paramecium* is an excellent system in which to study second messenger–mediated pathways because it is possible to combine biochemical with mutational analysis to dissect, at the molecular level, the pathways regulating motion (Haga et al., 1984; Hinrichsen et al., 1986; Saimi and Kung, 1987). Such pathways may be relevant to other motile and cellular processes similarly regulated by cAMP and/or Ca^{2+} (Johnson, 1985; Gilson et al., 1986; Rozdzial and Haimo, 1986).

A cilium or eukaryotic flagellum is composed of an axoneme, an enclosing membrane, and various associated proteins. The three-dimensional motion of the ciliary beat occurs through regulated interactions among the dynein ATPases, axonemal microtubules, and probably other proteins of the cilium (Gibbons, 1981; Satir, 1985). cAMP and Ca^{2+} must influence, directly or indirectly, the force-generating mechanism in order to modulate the basic characteristics of the motion. Biochemical regulation by second messengers is likely to entail multistep pathways, with Ca^{2+} activity being mediated by Ca^{2+}-binding proteins (Brokaw and Nagayama, 1985; Tash and Means, 1987), and cAMP functioning through stimulation of protein kinase and subsequent phosphorylation of target proteins (Brandt and Hoskins, 1980; Ishiguro et al., 1982; Opresko and Brokaw, 1983; Tash et al., 1984).

Cells made permeable by detergents have proven valuable in elucidating the functions of regulatory molecules in the control of cellular motility (Tash et al., 1984; Rozdzial and Haimo, 1986). We have used such a preparation to investigate the ciliary regulation of *Paramecium*. Paramecia made permeable by detergent lose their ATP, and motility ceases (Naitoh and Kaneko, 1973). However, the addition of MgATP, the substrate for ciliary dynein (Gibbons, 1963), reactivates the cilia to beat and the cells to swim (Naitoh and Kaneko, 1973). Such permeabilized, MgATP-reactivated cells, or models, imitate the living cell in their swimming behavior. The effects of various compounds on ciliary function can then be studied by addition to the extracellular medium and by observation of the effects on the speed and direction of the cell swimming behavior.

Here, we report that both cAMP and cGMP regulated the

ciliary motility of *Paramecium*, but that the effect of each was distinct. Both cyclic nucleotides increased the forward swimming speed of reactivated cells, but cAMP produced a right-handed helical swimming path, while cGMP produced left-handed helical motion. cAMP and cGMP also differed in their ability to antagonize Ca^{2+}-induced backward swimming; this may reflect differential integration, with the Ca^{2+} pathways mediating reversal. Our results support the hypothesis that cAMP and cGMP have similar stimulatory effects on ciliary beat frequency, but have different effects on beat direction.

Materials and Methods

Cell Culture

Paramecium tetraurelia, wild-type stock 51s, was grown axenically to late-logarithmic, early-stationary phase at 28°C in Soldo's crude medium (Van Wagtendonk, 1974), modified by adjusting concentrations of phosphatidyl-ethanolamine and phosphatidylcholine to 50 mg/liter and 125 mg/liter, respectively.

Preparation and Reactivation of Permeabilized Cells

Cells were harvested from growth medium and washed once in 4 mM K^+ solution (4 mM KCl, 1 mM $CaCl_2$, 0.01 mM EDTA, 1 mM 3-[N-morpho-lino]propanesulfonic acid, pH 7.2) at 20°C. They were then resuspended at 30,000–60,000 cells/ml in a Triton solution (0.01% Triton X-100, 20 mM KCl, 10 mM EGTA, 10 mM Tris-maleate, pH 7.0, 0–4°C) to permeabilize the cell membrane, and maintained on ice. When cells had ceased to swim and cilia to beat (10–20 min), the cells were washed once with the same volume of wash solution (50 mM potassium acetate, 10 mM EGTA, 10 mM Tris-maleate, pH 6.4, 0–4°C) and were then resuspended in wash solution and kept on ice. Cell washes and solution exchanges were performed by centrifugation for 2 min at 1,000 g in an HN-S centrifuge (International Equipment Co., Needham Heights, MA) using oil-testing centrifuge tubes.

Permeabilized cells were reactivated to swim at 30,000–50,000 cells/ml at room temperature (20–22°C) in a MgATP solution (4 mM $MgCl_2$, 4 mM ATP, 50 mM potassium acetate, 10 mM EGTA, 10 mM Tris-maleate, pH 6.4), plus other additions as noted in the text. For Figs. 1 and 2 and Table I, 1 mM dithiothreitol was included in Triton and wash solutions; it had no effect on the speed of reactivated cells. Cyclic nucleotides and other analogs were Na^+ salts (Sigma Chemical Co., St. Louis, MO). For Ca^{2+} solutions, $CaCl_2$ was added to obtain the desired free concentration at pH 6.4 using the equilibrium constants for H^+, Ca^{2+}, and Mg^{2+} with EGTA of Martell and Smith (1974), and with ATP of Chaberek and Martell (1959). Total $CaCl_2$ was 2.97 mM for 1.5 µM free Ca^{2+}. Cells in potassium phosphate buffer were permeabilized and reactivated as above, except all solutions contained 10 mM potassium phosphate in place of the Tris-maleate buffer.

Routine observation of the swimming behavior of models was made by reactivating cells to swim in depression slides and observing behavior with a Stereo Zoom 5 microscope (Bausch and Lomb, Rochester, NY) at 8- to 40-fold magnification. This microscope was used when determining the handedness of the helical swimming paths of cells; the image to the eye was not inverted. Dark-field photography of models was performed as described previously (Bonini et al., 1986).

We used three terms to describe the helical swimming paths of reactivated cells: narrow, wide, and tight. A narrow helix was of small amplitude (such as illustrated in Fig. 5 c), whereas a wide helix was of larger amplitude (for example, Fig. 5 d). In some cases (e.g., when swimming backward), cells swam in a helix of small amplitude with a large number of revolutions per distance traveled; this we called a tight helix (see Fig. 5, b and f).

Swimming Speed Measurements

The swimming velocity of reactivated cells was determined by analyzing digitized video data of swimming paths using the Expert Vision system of Motion Analysis Corp. (Santa Rosa, CA). Permeabilized cells were reactivated in 1.5-ml Beckman Microfuge tubes, and 25–50 µl of cells were transferred to the polished, spherical depression (18 mm in diameter, ~1 mm at maximum depth) of a 1″ × 3″ × 3-mm microscope culture slide. The cells were illuminated from the side with a Tensor lamp, such that the cells ap-
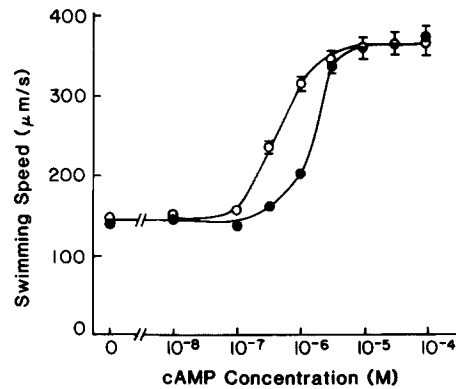

Figure 1. cAMP regulation of swimming speed of permeabilized cells; potentiation by PDE inhibitor IBMX. Paramecia were permeabilized with Triton X-100 and reactivated to swim in the presence of 4 mM MgATP and various concentrations of cAMP, in the presence (O) or absence (●) of 1 mM IBMX, a phosphodiesterase inhibitor. Data are presented as mean ± SEM. For points with no error bars, the SEM fell within the point.

peared white against a dark background of black paper beneath the slide. Cells were filmed from above with a CCD solid state camera (RCA Closed Circuit Video Equipment, Lancaster, PA) equipped with a Macrobel bellows lens and flat-field 75-mm macro lens (Spiratone Co., New York, NY). The camera was connected to a video monitor and cassette recorder.

In measurements of swimming speed for any one experiment, a constant volume of reactivated cells was filmed for each experimental point. The cells were filmed at the same time after addition to MgATP for all points of one experiment, typically 20–45 s after initiation of reactivation. Permeabilized cells could be reactivated for at least 60 min after permeabilization without decrement in swimming speed.

Linear velocities along the swimming paths were calculated from the video recordings by digitizing 1 or 2 s of data at 2 to 15 frames per second, depending upon the magnification of the filming and speed of the cells. Data are presented as the mean velocity ± SEM, where *n* is the number of cells, ranging from 50 to 300 per point. Cells were randomly selected for swimming speed measurements; at least 90% of the models reactivate upon addition of MgATP (Bonini et al., 1986). Results were generally quantified from at least two experiments and confirmed qualitatively many times.

Results

Regulation of Swimming Speed by cAMP and cGMP

Paramecia were permeabilized with 0.01% Triton X-100 and reactivated to swim by addition of MgATP. The basal swimming velocity was constant for a given preparation of models, and ranged from 100 to 200 µm/s. The speed of living cells is also variable, generally ranging from 500 to 1,000 µm/s (Bonini et al., 1986). The models responded to increasing concentrations of added cAMP with graded increases in swimming velocity (Fig. 1). Maximal speed was reached with ~10 µM cAMP, typically representing a two- to threefold increase over the basal velocity (mean increase was 2.4 ± 0.4 (SD), *n* = 12 independent experiments). Isobutylmethylxanthine (IBMX),[1] a cyclic nucleotide phosphodiesterase (PDE) inhibitor (Beavo et al., 1970), increased the sensitivity to cAMP concentrations between 0.5 and 5 µM (Fig. 1). The half-maximal effect on speed occurred at 0.65 µM cAMP in the presence of 1 mM IBMX and at ~2 µM in its absence;

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1. *Abbreviations used in this paper:* 8-Br, 8-bromo; IBMX, isobutylmethylxanthine.
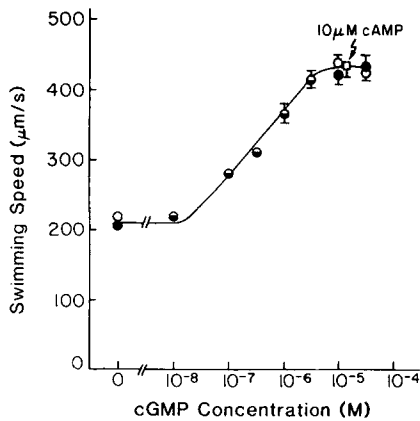
Figure 2. cGMP regulation of swimming speed of permeabilized cells. Cells were permeabilized with Triton X-100 and reactivated to swim with MgATP in the presence (○) or absence (●) of 1 mM IBMX and various concentrations of cGMP. cAMP addition at 10 μM (□), in the absence of added cGMP, resulted in a velocity increase similar to that of cGMP. Data are presented as mean ± SEM; where no error bars are shown, SEM was within the point.

the speed with saturating concentrations of cAMP was similar plus or minus IBMX. The potentiation of the response by IBMX suggests that the permeabilized cells have cAMP-PDE activity; an IBMX-sensitive PDE has been described in ciliary preparations from *Paramecium* (Gustin and Nelson, 1987).

cGMP was as effective as cAMP in increasing the swimming velocity of reactivated cells (Fig. 2). In fact, cGMP was consistently effective at lower concentrations than cAMP, causing a clear stimulation at 0.1 μM. The response was unaltered by the addition of IBMX, suggesting that cGMP was not significantly hydrolyzed by IBMX-sensitive PDE activity of the models. Maximal velocity was similar with saturating
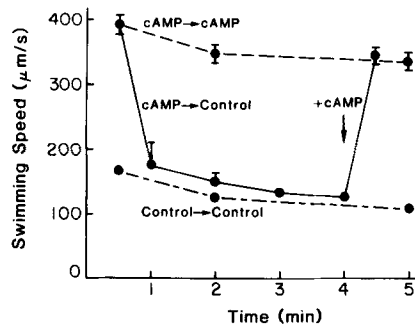
Figure 3. cAMP stimulation of swimming; reversal by dilution of cAMP. Permeabilized cells were reactivated to swim at 100,000 cells/ml in 4 mM MgATP with (*cAMP*) or without (*control*) 10 μM cAMP, and swimming speed was measured 30 s after initiation of reactivation. At 30 s, cells were diluted 10-fold into the same solution (*control → control*; *cAMP → cAMP*) or from 10 μM cAMP into a solution without cAMP (*cAMP → control*) to reverse the stimulation by dilution of the cAMP concentration. At 4 min, the cAMP response was reinitiated in the cAMP → control cells by addition of 50 μl of 200 μM cAMP to 1 ml of cells (*arrow*), for a final additional cAMP concentration of 9.5 μM. Swimming velocity was measured 30 s later. Data are presented as mean ± SEM; where no error bars are indicated, the SEM fell within the point.

Table I. Cyclic Nucleotides Increase Forward Swimming Speed of Permeabilized Cells

Addition	Concentration	Swimming speed	Relative activity
	μM	μm/s	
None (control)	–	161 ± 4	100%
cAMP	10	365 ± 11	227%
8-Br-cAMP	10	360 ± 10	224%
8-Br-cGMP	10	364 ± 10	226%
N⁶-monobutyryl-cAMP	10	252 ± 9	157%
N⁶-benzoyl-cAMP	10	280 ± 10	174%
Adenosine	100	162 ± 5	101%
5'-AMP	100	159 ± 6	99%
5'-GMP	100	160 ± 6	99%
NaCl	100	161 ± 6	100%

Permeabilized cells were reactivated to swim by addition of 4 mM MgATP in the presence or absence of the indicated cyclic nucleotides or analogs. Swimming speeds are presented as mean ± SEM.

(10 μM) concentrations of cGMP and cAMP, although the half-maximal concentration for cGMP, 0.55 μM, was slightly lower than that for cAMP (with 1 mM IBMX).

## Reversibility of the Cyclic Nucleotide Stimulation

The fast swimming response of living cells is normally transient (Naitoh and Eckert, 1969), and is thus reversible. To determine whether this aspect of the physiological response was present in our permeabilized cell preparation, we reactivated models in the presence of 10 μM cAMP, then diluted to 1 μM cAMP and observed the effect of the dilution on swimming speed. A concentration of 1 μM was near threshold for stimulation of swimming by cAMP in the absence of IBMX (see Fig. 1). The stimulation by cyclic nucleotide was completely reversible (Fig. 3). Upon dilution of cAMP, reactivated cells reduced their swimming speed rapidly to the control (without cAMP) level, and were restimulated to maximal speed by subsequent addition of cAMP. Cell transfer per se did not reduce velocity since transfers from 10 μM cAMP into 10 μM cAMP did not significantly alter swimming speed.

The decay of the cAMP response demonstrated here probably reflects a combination of reversal due partially to dilution of the cAMP (within 30 s), and partially to hydrolysis of the residual 1 μM cAMP by the models (1–3 min; data not shown), which further reduced the cAMP concentration.

The response to 10 μM cGMP was also reversible upon dilution to 0.05 μM, near the predicted threshold concentration from the dose-response curve (see Fig. 2; data not shown).

Swimming Response to Other Nucleotide Derivatives

cAMP and cGMP may act by stimulating cAMP- and cGMP-dependent protein kinase activities, respectively (Greengard, 1978). Alternatively, they might both act on a single kinase activity. Several cyclic nucleotide derivatives that stimulate the cyclic nucleotide-dependent protein kinases of *Paramecium* in vitro (Miglietta, 1987; Mason, 1987) were tested for stimulation of fast swimming in models. Compounds derivatized at the C-8 position, (8-bromo-cAMP [8-Br-cAMP], 8-Br-cGMP, Table I; 8-azido-cAMP, 8-thio-
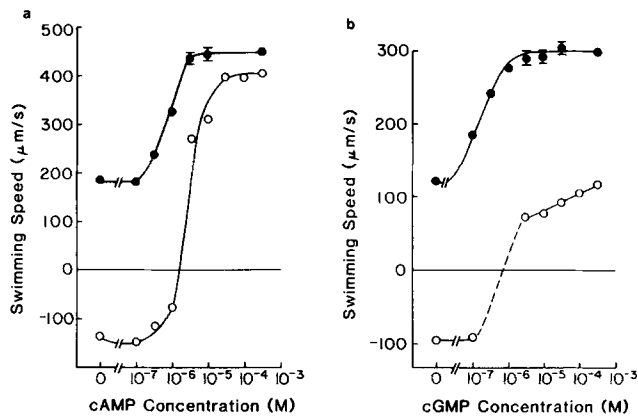
Figure 4. Antagonism by cyclic nucleotides of Ca^{2+}-induced backward swimming; increased effectiveness of cAMP over cGMP. (a) Concentration dependence of cAMP in the presence (O) and absence (●) of 1.5 μM free Ca^{2+}. A negative swimming velocity indicates backward swimming. (b) Concentration dependence of 8-Br-cGMP in the presence (O) and absence (●) of 1.5 μM free Ca^{2+}. Since the response of the cells to Ca^{2+} and 0.5 or 1.0 μM 8-Br-cGMP showed variability (cells whirled in place or moved forward or backward), this transition between backward and forward swimming is represented by a dashed line. a and b represent data from independent experiments. Swimming speed data are presented as mean ± SEM. For points without error bars, the SEM was within the point.

methyl-cAMP, data not shown) were as effective as cAMP at concentrations of 10 μM. The membrane-permeant analogs N^6-monobutyryl-cAMP and N^6-benzoyl-cAMP, which increase the swimming speed of living cells (Bonini et al., 1986), also produced accelerated swimming in models, but at 10 μM were not as effective as cAMP (Table I). Addition of 1 mM IBMX did not alter these results markedly (data not shown).

That 8-Br-cGMP was as effective as cGMP or cAMP at increasing the swimming speed of models (Table I; also Fig. 4 b) was notable. In vitro, 8-Br-cGMP is a poor activator of the cAMP-dependent protein kinases of *Paramecium* (half-maximal activation requires $>10^{-4}$ M [Mason, 1987]), although it potently activates the cGMP-dependent protein kinase (half-maximal activation at 5×10^{-8} M [Miglietta, 1987]). This is in contrast to cGMP itself, which can activate the cAMP-dependent protein kinases in vitro, although not as effectively as cAMP (Mason, 1987). Because of this biochemical specificity, we generally used 8-Br-cGMP rather than cGMP in subsequent studies, although similar results on the behavior of models were obtained with cGMP.

The stimulation of forward swimming was specific for cyclic nucleotides: 100 μM adenosine, 5'-AMP, or 5'-GMP (the metabolic degradation products of cAMP and cGMP, respectively) had no effect on the swimming velocity of models (Table I).

Interactions between Cyclic Nucleotides and Ca²⁺

Electrophysiological and genetic evidence indicates that *Paramecium* uses Ca^{2+} as an intraciliary signal for backward swimming (Eckert et al., 1976; Kung and Saimi, 1982; Saimi and Kung, 1987). Ca^{2+} action directly upon the axoneme is suggested in permeabilized cells, which reverse ciliary beat

Table II. Different Effects of cAMP and cGMP on the Helicity of Swimming Paths of Reactivated Cells

Addition	Swimming speed	Helix handedness
	μm/s	
*In Tris-maleate buffer		
None (control)	214 ± 7	Right
cAMP	443 ± 18	Right
8-Br-cGMP	427 ± 15	Wide helices (right or left)
cAMP + 8-Br-cGMP	444 ± 15	Right
Ca^{2+}	−183 ± 6	Right
Ca^{2+} + cAMP	349 ± 15	Right
Ca^{2+} + 8-Br-cGMP	137 ± 6	Left
Ca^{2+} + cAMP + 8-Br-cGMP	414 ± 17	Right
‡In phosphate buffer		
None (control)	30 ± 1	−
cAMP	425 ± 10	Right
cGMP	337 ± 9	Left
cAMP + cGMP	443 ± 12	Right

* Cells were permeabilized and reactivated by the standard procedure (in a 10-mM Tris-maleate buffer). For speed measurements, cyclic nucleotides were present at 40 μM, Ca^{2+} at 1.5-μM free concentration. Swimming direction was forward except in Ca^{2+} alone when direction was backward, indicated by the negative speed. Helix observations represent the handedness of 90–100% of 25–40 cells scored within 1 min of initiation of reactivation from each of at least three reactivation experiments. For cGMP in the absence of Ca^{2+}, the helical response ranged from 100% left-handed helices, to 87% right- and 13% left-handed helices. Although 8-Br-cGMP was used in these experiments, the same behavioral response was seen with cGMP (data not shown).
‡ Cells were permeabilized and reactivated as above, but with a 10-mM potassium phosphate buffer replacing the Tris-maleate buffer. Cyclic nucleotides were present at 100 μM, IBMX at 1 mM. Observations of the helical paths represent the handedness of 100% of 30 cells from each of three reactivation experiments. Helices were narrow, as in Fig. 5 c, but mirror images for cAMP and cGMP additions. Control cells reactivated too poorly to distinguish a helical path. Swimming speeds are presented as mean ± SEM.

orientation and swim backward in the presence of micromolar Ca^{2+} concentrations (Naitoh and Kaneko, 1972). Our models demonstrated this response: as the free Ca^{2+} concentration was increased from 10^{-8} to 10^{-4} M, forward swimming slowed at 0.5 μM Ca^{2+}, and the models swam backward in the presence of 1–5 μM Ca^{2+}. We investigated the interactions between Ca^{2+} and cyclic nucleotides by maintaining the free Ca^{2+} concentration at 1.5 μM, which caused maximal backward swimming in the absence of cyclic nucleotide, and then varying the cyclic nucleotide concentration. Ca^{2+}-induced backward swimming was overcome by cAMP and cGMP, although the response of the cells in the presence of cAMP and Ca^{2+} was not the same as in the presence of cGMP and Ca^{2+}.

The response to cAMP (+ 1 mM IBMX) in the presence and absence of Ca^{2+} is shown in Fig. 4 a. As in Fig. 1, addition of cAMP to models increased the forward swimming velocity. Ca^{2+} at 1.5 μM induced backward swimming; however, with addition of increasing concentrations of cAMP, cells first reduced their backward swimming speed, then reversed swimming direction from backward to forward. The concentration dependence curve for cAMP was shifted about fourfold in the presence of Ca^{2+}, such that the half-maximal concentration for the forward speed increase was 3 rather than 0.8 μM. The maximal forward velocity was

~90% of that attained with cAMP in the absence of Ca^{2+}. Thus, cAMP stimulated fast forward swimming of models in the continued presence of a Ca^{2+} concentration that would normally cause ciliary reversal. Therefore, cAMP antagonized the ability of Ca^{2+} to induce backward swimming.

The response to cGMP in the presence of Ca^{2+} was not the same as the response to cAMP (Fig. 4 b). As in Fig. 2, 8-Br-cGMP in the absence of Ca^{2+} stimulated forward swimming 2.5-fold over the basal speed. Addition of 1.5 μM Ca^{2+} induced backward swimming, but increasing the concentration of 8-Br-cGMP from 1 to 500 μM, in the continued presence of Ca^{2+}, reversed swimming direction such that cells swam forward, albeit with low velocity: 25–40% of the speed with 8-Br-cGMP alone. Similar results were obtained with cGMP as with the 8-Br-cGMP derivative (data not shown). Therefore, although cGMP antagonized the ability of Ca^{2+} to induce backward swimming, models did not attain a forward speed comparable to that attained with cGMP in the absence of Ca^{2+}. The antagonism was specific to cyclic nucleotides: 500 μM 5'-AMP, 5'-GMP, or adenosine had no effect on Ca^{2+}-induced backward swimming (data not shown).

Therefore, both cAMP and cGMP could overcome the ability of Ca^{2+} to induce backward swimming, but the responses to the two cyclic nucleotides were distinct in the ability to cause fast forward swimming in the continued presence of Ca^{2+}. A closer examination revealed a striking difference between the detailed swimming behavior of models with cAMP and cGMP in the presence and absence of Ca^{2+}.

Different Effects of cAMP and cGMP on the Helical Swimming Path

Living paramecia swim in a helical path that is right- or left-handed depending upon the species of Paramecium (Bullington, 1930). Paramecium tetraurelia, the species used in these studies, swims forward in a left-handed helix. Our reactivated models swam forward in a right-handed helix (Table II [*]; Fig. 5 a), opposite to that of the living cell. Since helical handedness primarily reflects ciliary beat direction (Bullington, 1930; Párducz, 1962, 1967; Machemer, 1972; see Discussion), some aspect of the permeabilization procedure presumably shifted the beat direction (see Discussion).

In the presence of cAMP, permeabilized cells swam forward rapidly in a right-handed helix (Table II [*]; Fig. 5 c). In the presence of cGMP, the permeabilized cells also swam fast forward, but the swimming paths were wide helices, either left- or right-handed (Table II [*]; Fig. 5 d). This suggested that cGMP was shifting ciliary beat direction to adjust the swimming path from a right- to a left-handed helix.

The different effects of the cyclic nucleotides were more striking when we replaced the Tris-maleate buffer in the permeabilization and reactivation solutions with a potassium phosphate buffer. It had been observed that the cAMP-dependent protein kinases of Paramecium demonstrate greater cyclic nucleotide stimulation in phosphate than other buffers (Mason, 1987). Therefore, we developed models prepared in potassium phosphate buffer to provide a system with greater signal/noise for biochemical studies, as well as mutant screening; other characteristics of the preparation will be reported elsewhere (Bonini, N. M., and D. L. Nelson, manuscript in preparation).
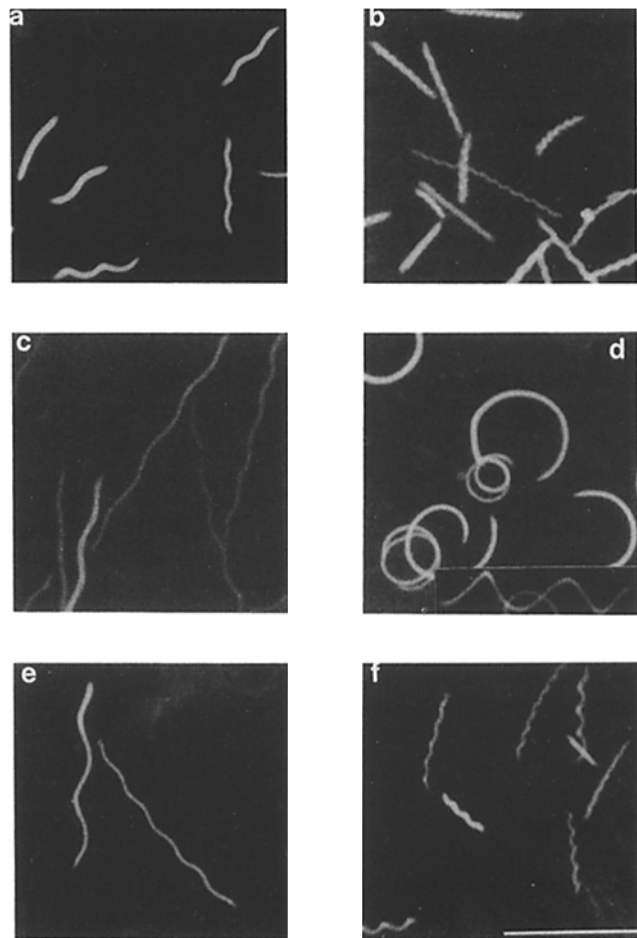
Figure 5. Different helical swimming paths of permeabilized cells with cAMP, cGMP, and Ca^{2+}. Permeabilized cells were reactivated in a depression slide below a camera while illuminated from the side with a lamp. In these 5-s time exposure photographs, the cells made swimming paths that appear white against the dark background. Tris-maleate solutions were used. (a) Control cells (MgATP alone). The waviness of the traces is characteristic of the helical swimming path of cells. (b) Ca^{2+}-induced backward swimming (1.5 μM free Ca^{2+}). Cells swam backward in tight helices. (c) cAMP (80 μM). Cells swam forward in narrow helices. (d) cGMP (80 μM; the 8-Br-cGMP derivative was used). Most cells swam in wide helices. (inset) The wide helix of a forward-moving cell. (e) Ca^{2+} + cAMP (1.5 μM Ca^{2+}, 80 μM cAMP). Cells swam forward in helices similar to those with cAMP alone. (f) Ca^{2+} + cGMP (1.5 μM Ca^{2+}, 80 μM 8-Br-cGMP). Cells swam in tight helices similar to the Ca^{2+}-backward helix except that these cells were moving forward. Bar, 1 mm.

Models reactivated in phosphate buffer in the presence of MgATP alone swam very poorly (Table II [‡]). cAMP addition caused them to swim forward rapidly in a narrow right-handed helix; however, cGMP addition caused them to swim forward rapidly in a narrow left-handed helix (Table II [‡]). Thus, replacement of the Tris-maleate buffer with phosphate allowed a clearer observation of the response to cGMP as a shift in the motion to a left-handed helix. In either buffer, if both cAMP and cGMP were added together at concentrations eliciting maximal responses for each alone, the behavioral response was similar to the cAMP pattern: fast for-

ward swimming in a narrow right-handed helix (Table II, [* and ‡]).

Addition of Ca^{2+} to Tris-maleate models caused backward swimming in a helix similar to the backward response of *P. tetraurelia* in vivo (Bullington, 1930): the models moved backward in a tight right-handed helix (Table II [*]; Fig. 5 b). Increasing the concentration of cAMP in the presence of Ca^{2+} caused cells to change swimming direction from backward to forward (see Fig. 4 a). In doing so, models moved forward at low cAMP concentrations in a left-handed helix, with greater concentrations in a wide helix, then, with >10 μM cAMP, in a right-handed helix (Table II; Fig. 5 e; data not shown).

cGMP addition resulted in a different response. In the presence of Ca^{2+} and increasing concentrations of cGMP, the models slowed backward swimming, whirled counterclockwise in place, then moved forward, still rotating counterclockwise. The result was a very tight left-handed helix that resembled the backward swimming helix, except that these cells were moving forward (Table II [*]; Fig. 5 f). The rate of forward progression was typically low (see Fig. 4 b), although the cells appeared to be spinning rapidly counterclockwise while moving forward.

In the presence of Ca^{2+}, cGMP, and cAMP, the helix and speed were again characteristic of the cAMP response: the cells swam forward rapidly in a narrow right-handed helix (Table II [*]).

Discussion

We report the novel finding that the ciliary motility of *Paramecium* is differentially regulated by cAMP and cGMP. The use of detergent-permeabilized, MgATP-reactivated cells allowed experimental access to the axonemal apparatus, while eliminating complicating effects of feedback onto the membrane potential by second messengers. Both cGMP and cAMP stimulated the forward swimming of permeabilized cells with similar sensitivity, but the effects on the helical swimming path of cells were strikingly different, as were the abilities to antagonize the effect of Ca^{2+} on ciliary motion. Although many cellular processes demonstrate regulation by cAMP, there are relatively few known examples of regulation by cGMP (Goldberg and Haddox, 1977; Lincoln and Corbin, 1983). Our results suggest that cGMP can act as a second messenger, distinct from cAMP, in the regulation of ciliary motion.

In *Paramecium*, swimming speed and direction are primarily a reflection of the ciliary beat frequency and direction of the ciliary power stroke (Eckert et al., 1976). These are coregulated in vivo and coupled to the membrane potential of the cell (Machemer, 1974; Machemer and Eckert, 1975). Studies using simultaneous recording of the membrane potential, ciliary beat frequency, and direction of the ciliary power stroke (Machemer, 1974; Machemer and Eckert, 1975) have provided insight into the coupling process. During basal forward locomotion, the effective stroke of the beat cycle is directed toward the posterior of the cell, but obliquely to the right (5 o'clock, with the anterior of the cell defined as 12 o'clock). The cell swims forward while rotating to the left along its anterior–posterior axis, resulting in a left-handed helix (Jennings, 1906; Bullington, 1930). Membrane

hyperpolarization produces faster forward swimming in a left-handed helix with both a two- to threefold increase in ciliary beat frequency and a shift of beat direction from 5 o'clock toward 6 o'clock (Machemer, 1974; Machemer and Eckert, 1975). Upon membrane depolarization, both beat frequency and direction are again modulated: frequency increases, while orientation shifts from a posteriorly directed beat to an anteriorly directed beat (5 o'clock toward 1 o'clock; Machemer, 1974; Machemer and Eckert, 1975). As a result, the cell swims fast backward. Ca^{2+} and cAMP act as second messengers, coupling events at the membrane to those of the ciliary axoneme: Ca^{2+} triggering the ciliary events of backward swimming (Naitoh and Kaneko, 1972; Eckert et al., 1976; Kung and Saimi, 1982) and cAMP increasing in association with fast forward swimming (Bonini et al., 1986). Permeabilized cells respond to Ca^{2+} by swimming backward (Naitoh and Kaneko, 1972) and to cAMP by swimming forward faster (Nakaoka and Ooi, 1985; Bonini et al., 1986; this report).

Regulation of the swimming speed of a cell or permeabilized model could be the result of modulation of ciliary beat frequency, beat direction, or both. Measurements of swimming speed are resultant of both factors. It is probable that an increase in beat frequency is part of the fast swimming speed response of models to cyclic nucleotide addition. In *Paramecium*, graded increases in ciliary beat frequency accompany increases in the speed of intact (Nakaoka et al., 1983) and permeabilized cells (Toyotama and Nakaoka, 1979; Nakaoka et al., 1984), and are elicited upon microinjection of cAMP or 8-Br-cGMP into living cells (Hennessey et al., 1985). cAMP is known to regulate the beat frequency of sperm flagella (Lindemann, 1978; Tash et al., 1984) and of *Mytilus* gill cilia (Murakami, 1983; Stommel and Stephens, 1985).

The differences in helical paths of models in cAMP or cGMP reported here suggest the two cyclic nucleotides also affect, differentially, the direction of the ciliary power stroke. Paramecia that swim in left- or right-handed helices differ in that the direction of the ciliary power stroke is toward 7 o'clock for a right-handed helix and toward 5 o'clock for a left-handed helix (Párducz, 1962, 1967; Dryl and Grębecki, 1966; Machemer, 1972). We interpret the differences in the helical handedness of the motion of reactivated cells to also result from differences in beat direction, although we have not directly visualized beat direction.

Our models reactivated in MgATP alone swam in a right-handed helix (Fig. 6 A). The conditions we used for making cells permeable apparently altered the effective stroke from a 5 o'clock to a 7 o'clock position, changing the left-handed helix of the intact cell to a right-handed one of the reactivated cell. This fortuitous change to right-handed motion may have allowed us to see aspects of ciliary regulation not apparent in other reported preparations, which typically reactivate to swim in left-handed helices (Naitoh and Kaneko, 1973; Nakaoka and Ooi, 1985). cAMP addition increased swimming speed without changing the helical handedness from the right-handed motion of the control (Fig. 6 B). We interpret the effects of cAMP as (a) an increase in beat frequency and (b) a bias toward the 7 o'clock ciliary beat direction. cGMP also increased swimming speed, but with a shift from a right-handed to a left-handed helix. We suggest cGMP also
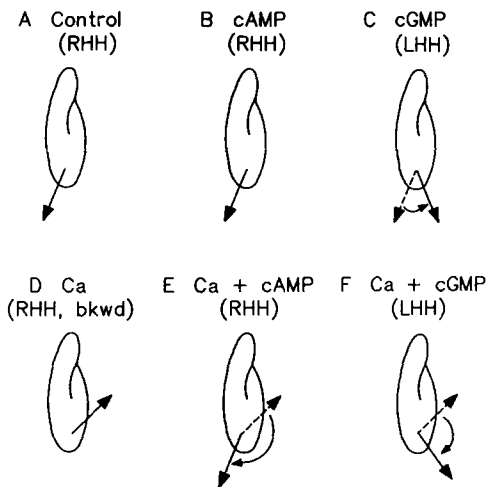
Figure 6. Effects of cyclic nucleotides and Ca²⁺ on ciliary beat direction of permeabilized cells. Our interpretation of ciliary beat direction based on observations of the helical swimming paths of reactivated cells (see Table II). Arrows represent the direction of the power stroke of the cilia that cover the cell body, from the perspective of looking down on the cell. Cells swim forward except in condition D, with Ca²⁺ and no added cyclic nucleotide. In vivo, *Paramecium* swims forward with the beat direction at 5 o'clock (Machemer, 1974). (*A*) Control (MgATP alone). Beat direction is 7 o'clock; (*B*) cAMP, beat direction is 7 o'clock; (*C*) cGMP, beat direction shifts toward 5 o'clock; (*D*) Ca²⁺, beat direction is shifted toward 1 o'clock, and the cell swims backward; (*E*) Ca²⁺ + cAMP, beat direction shifts from 1 o'clock to 7 o'clock; (*F*) Ca²⁺ + cGMP, beat direction shifts from 1 o'clock to 4 or 5 o'clock. *RHH*, right-handed helix; *LHH*, left-handed helix; *bkwd*, backward swimming of the cell.

increases beat frequency, but favors a left-handed helix by adjusting beat direction from the control orientation of 7 o'clock toward a 5 o'clock position (Fig. 6 *C*).

In the Tris-maleate buffer, cells in cGMP swam in wide but distinct helices. This behavior may represent a transition stage between helices of different handedness, since cells in the presence of Ca²⁺ and increasing concentrations of cAMP went through a similar motion when changing from forward swimming in a left- to a right-handed helix. When models were permeabilized and reactivated in phosphate buffer, the effects of cyclic nucleotides on the helical path were even more striking: models swam in narrow right- and left-handed helices in cAMP and cGMP, respectively. The slow basal velocity of phosphate models in the absence of cAMP or cGMP indicates that cyclic nucleotide may be required for ciliary motion.

Ca²⁺-induced backward swimming of models (Fig. 6 *D*) was antagonized by cAMP and cGMP, although the behavioral responses were different with the two nucleotides. Backward swimming is accompanied by a shift in beat direction toward 1 o'clock (Naitoh and Kaneko, 1972; Machemer, 1974). Our observations suggest that cAMP overcame Ca²⁺-induced backward swimming, restoring beat orientation clockwise from 1 o'clock, through 5 o'clock, to the 7 o'clock position (Fig. 6 *E*). Although cGMP reversed backward swimming, the effect on the helix suggests that cGMP in the presence of Ca²⁺ favored a ciliary position of 4 or 5 o'clock

(Fig. 6 *F*). The reduced forward speed in the presence of Ca²⁺ and cGMP may reflect a slow beat frequency, or may result from an effect on beat orientation preventing rapid forward progress.

The different effects of cAMP and cGMP in models suggest distinct roles in vivo. cAMP likely acts through a pathway functional during membrane hyperpolarization (Bonini et al., 1986). Hyperpolarization increases intracellular cAMP levels 1.5- to 2-fold and doubles swimming speed. IBMX and membrane-permeant analogs of cAMP mimic hyperpolarization by inducing the fast swimming response. Although intact *P. tetraurelia* swim fast in a left-handed helix, membrane hyperpolarization does shift ciliary beat direction from the 5 o'clock position toward 6 o'clock, in conjunction with the ciliary beat frequency increase (Machemer, 1974; Machemer and Eckert, 1975). A clockwise rotation of beat direction is consistent with the effects of cAMP on the helical handedness of our models.

cGMP levels do not vary in parallel with cAMP in vivo: cGMP increases under the strong depolarizing conditions (Schultz et al., 1986) associated with prolonged backward swimming (Naitoh, 1968). This is probably due to activation of the Ca²⁺-dependent guanylate cyclase (Schultz and Klumpp, 1984). The levels of cGMP measured in vivo are high in *Paramecium* (Schultz et al., 1986; Bonini, N. M., and D. L. Nelson, unpublished observations), comparable to those of cAMP (Schultz et al., 1984; Bonini et al., 1986). This further supports a physiological role for cGMP. Since ciliary beat frequency also increases with fast backward swimming (Machemer, 1974; Machemer and Eckert, 1975), cGMP may regulate frequency during depolarization, as cAMP does during hyperpolarization. Detergent permeabilization of cells may have disrupted compartmentalization of these effects. Alternatively, or in addition, cGMP may function in the time-dependent adaptation of ciliary beat direction to depolarizing (Naitoh, 1968) or hyperpolarizing (Machemer, 1974; Machemer and Eckert, 1975) stimuli, as it apparently favors the basal forward swimming position of 5 o'clock. In nature, dynamic regulation by second messengers probably consists of consecutive waves of Ca²⁺ and cyclic nucleotides serving to transiently modify ciliary motion on a rapid time scale (Machemer, 1974; Machemer and Eckert, 1975). Regardless of the function of cGMP in vivo, our data indicate that regulation of motility by cGMP is through pathways that are functionally, thus probably biochemically, distinct from those of cAMP.

The ciliary targets of cAMP and cGMP that mediate the actions of the cyclic nucleotides probably include cyclic nucleotide–dependent protein kinases (Greengard, 1978). Cilia contain relatively high levels of both cAMP- and cGMP-dependent protein kinases, which discriminate strongly between cAMP and 8-Br-cGMP (Mason, 1987; Miglietta, 1987). Cilia and axonemes also contain substrates for these kinases (Lewis and Nelson, 1981; Eistetter et al., 1983). Our permeabilized cell preparation affords the opportunity to identify targets of kinase regulation under the physiological conditions of a motile response. We are characterizing substrates phosphorylated by cAMP and cGMP in models (Bonini, N. M., and D. L. Nelson, manuscript in preparation); those differentially phosphorylated may function in unique aspects of the cAMP and cGMP pathways, such as the con-

trol of ciliary beat direction. The fast swimming response in models may be achieved by modulation of dynein ATPase activity. Dyneins may be direct targets of regulation by phosphorylation (Piperno and Luck, 1981; Tash and Means, 1982; Travis, 1987); alternatively, ATPase activity may be modulated indirectly through alteration of the function of other target phosphoproteins. The antagonistic effects of Ca^{2+} and cyclic nucleotides suggest integration at the molecular level among the signal transduction pathways. One site of integration between cAMP and Ca^{2+} may be phosphoprotein phosphatase (Tash and Means, 1987).

Since in *Paramecium* there exists the potential to isolate mutants biochemically altered in the behavioral responses to cAMP and cGMP, we are currently using genetics to test our predictions and dissect these signal transduction pathways. Other ciliary or flagellar systems demonstrate modulation by cAMP (Garbers et al., 1971; Lindemann, 1978; Murakami, 1983; Stommel and Stephens, 1985) and cGMP (Lindemann, 1978; Hansbrough et al., 1980), but the effects of cGMP on axonemal regulation have been largely unexplored. Although the cilium is a force-generating mechanism, highly specialized for a particular type of motion, other types of motile processes, such as actin-myosin (Adelstein and Eisenberg, 1980), retinal elongation (Gilson et al., 1986), and melanophore movement (Lynch et al., 1986; Rozdzial and Haimo, 1986), among others, are also regulated by cAMP and/or Ca^{2+}, and may use similar motility factors or principles of regulation. Thus, the elucidation of such regulation to the level of effector molecules in a cilium may be of fundamental relevance to other types of cellular motility.

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