Regulation of ciliary adenylate cyclase by Ca^{2+} in *Paramecium*

Michael C. GUSTIN and David L. NELSON*

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin–Madison, Madison, WI 53706, U.S.A.

In the ciliated protozoan *Paramecium*, Ca^{2+} and cyclic nucleotides are believed to act as second messengers in the regulation of the ciliary beat. Ciliary adenylate cyclase was activated 20–30-fold (half-maximal at $0.8 \mu\text{M}$) and inhibited by higher concentrations ($10\text{--}20 \mu\text{M}$) of free Ca^{2+} ion. Ca^{2+} activation was the result of an increase in V_{max} , not a change in K_m for ATP. The activation by Ca^{2+} was seen only with Mg^{2+}ATP as substrate; with Mn^{2+}ATP the basal adenylate cyclase activity was 10–20-fold above that with Mg^{2+}ATP , and there was no further activation by Ca^{2+} . The stimulation by Ca^{2+} of the enzyme in cilia and ciliary membranes was blocked by the calmodulin antagonists calmidazolium (half-inhibition at $5 \mu\text{M}$), trifluoperazine ($70 \mu\text{M}$) and W-7 ($50\text{--}100 \mu\text{M}$). When ciliary membranes (which contained most of the ciliary adenylate cyclase) were prepared in the presence of Ca^{2+} , their adenylate cyclase was insensitive to Ca^{2+} in the assay. However, the inclusion of EGTA in buffers used for fractionation of cilia resulted in full retention of Ca^{2+} -sensitivity by the ciliary membrane adenylate cyclase. The membrane-active agent saponin specifically suppressed the Ca^{2+} -dependent adenylate cyclase without inhibiting basal activity with Mg^{2+}ATP or Mn^{2+}ATP . The ciliary adenylate cyclase was shown to be distinct from the Ca^{2+} -dependent guanylate cyclase; the two activities had different kinetic parameters and different responses to added calmodulin and calmodulin antagonists. Our results suggest that Ca^{2+} influx through the voltage-sensitive Ca^{2+} channels in the ciliary membrane may influence intraciliary cyclic AMP concentrations by regulating adenylate cyclase.

INTRODUCTION

Both the orientation and the frequency of the ciliary beat in the protozoan *Paramecium* are known to be regulated by the electrical potential across the excitable surface membrane [1–3]. Depolarization induced by any of several external stimuli results in transient reversal in the direction of the ciliary beat, increased beat frequency, and thus fast, backward, swimming. Hyperpolarization induced, for example, by mechanical stimulation at the cell's posterior, results in increased beat frequency with normal orientation, and thus faster forward swimming. Studies with permeabilized cells have shown that Ca^{2+} is the likely mediator of the depolarization-induced ciliary reversal [4,5] and may also influence beat frequency [5]. Increasing the free Ca^{2+} concentration from $0.01 \mu\text{M}$ to $1 \mu\text{M}$ reverses the swimming direction of detergent-permeabilized cells ('models') [4–6]. Recent studies [6–9] have shown that cyclic AMP mediates increased beat frequency and may also influence beat orientation. The addition of micromolar concentrations of cyclic AMP increases the swimming speed of permeable models ([6,8,9]; N. M. Bonini & D. L. Nelson, unpublished work) and microinjection of cyclic AMP increases the ciliary beat frequency [7].

A central problem in the study of ciliary beat regulation in *Paramecium* is how the intracellular concentrations of these two putative second messengers, Ca^{2+} and cyclic AMP, are connected to the cell's membrane potential. Depolarization-activated Ca^{2+} channels [1,3] localized in the ciliary membrane [10,11] certainly play a major role in regulating intraciliary Ca^{2+}

concentration. Nothing is yet known about the mechanism by which Ca^{2+} is removed from the intraciliary space after a depolarization. Since the localization of the Ca^{2+} channels to the cilium suggests that regulation of second-messenger concentration could be locally controlled, we investigated the possibility of local control of cyclic AMP concentrations by studying the regulation of adenylate cyclase (and of PDE) in ciliary fractions. Our results demonstrate clear modulation of adenylate cyclase activity by Ca^{2+} , suggesting at least one point of interaction of these two second-messenger systems thought to participate in ciliary regulation.

EXPERIMENTAL

Paramecium tetraurelia strain 51S, grown in Cerophyl medium [12] supplemented with $5 \mu\text{g}$ of stigmaterol/ml and buffered to pH 6.8 with sodium phosphate, was used for all experiments. Cells were grown to early stationary phase before harvest. Deciliation and preparation of ciliary fractions were carried out as described by Adoutte *et al.* [13]. P1 and S1 refer to pellet and supernatant fractions obtained by centrifugation immediately after hypo-osmotic shock of cilia. G1, G2 and G3 refer to sucrose-density-gradient fractions: G1 (ciliary membrane vesicles) from the 20%/45% boundary, G2 (incompletely demembrated cilia) from the 45%/55% boundary, and G3 (mainly axonemes) from the 55%/66% boundary. All sedimentable fractions were washed in 25 mM-Tris/acetate, pH 7.0, with or without 0.1 mM-EGTA, before enzyme assays. Protein was measured by the procedure of Lowry *et al.* [14] with bovine serum albumin as standard.

Abbreviations used: PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine.

* To whom reprint requests should be sent.

Cyclic AMP PDE was assayed by measuring the conversion of cyclic [2,8-³H]AMP into labelled AMP [15]. The assay medium contained 50 mM-Tris/acetate (pH 7.6), 2 mM-magnesium acetate, 10⁵ c.p.m. of labelled cyclic AMP, various concentrations of unlabelled cyclic AMP, 1 mg of bovine serum albumin/ml and 1–5 µg of ciliary protein in a final volume of 50 µl at 25 °C. Incubations were started by the addition of substrate and quenched after 5 min by addition of 50 µl of 10% (v/v) trichloroacetic acid. Inclusion of 1 mM-5'-AMP to inhibit enzymic breakdown of the labelled AMP formed did not change the measured activity (results not shown) and therefore was not routinely done. Product was separated from substrate on Florisil columns by the procedure of Sinha & Colman [16]. Recovery of AMP was estimated (from the recovery of authentic labelled AMP) to be 85–90% in all experiments.

Adenylate cyclase activity was assayed by measuring the conversion of [α -³²P]ATP into labelled cyclic AMP by membrane-bound or soluble enzyme. Incubations were conducted for 10 min at 25 °C with 10–20 µg of protein from the indicated ciliary fraction in a final volume of 50 µl. The assay medium contained 25 mM-Tris/acetate (pH 7.0), 5 mM-magnesium acetate or -manganous acetate, 5 mM-phosphocreatine, 50 units of creatine kinase/ml, 1.5 µM-cyclic AMP, 1×10^6 – 2×10^6 c.p.m. of labelled ATP, various concentrations of added unlabelled ATP (usually 40 µM) and other additions as indicated in Figures and Tables. Reactions were started by the addition of ciliary-fraction protein and stopped by the addition of 150 µl of 'stop solution', containing 1.5 mM-cyclic AMP, 38 mM-ATP (unlabelled) and 1.3% SDS. To estimate recovery, 10⁵ c.p.m. of cyclic [2,8-³H]AMP was routinely included in the stop solution. The method of Salomon *et al.* [17] was used to separate the cyclic [³²P]AMP generated during the enzymic reaction. Briefly, Dowex AG50W-X4 and alumina columns mounted in series were used to purify labelled cyclic AMP; recovery of cyclic AMP was 80–90% with this method.

Guanylate cyclase activity was assayed by measuring the conversion of [α -³²P]GTP into labelled cyclic GMP. Incubation conditions and concentrations of assay buffer and quench-solution constituents were identical with those described above for the adenylate cyclase, except that guanine nucleotides replaced the corresponding adenine nucleotides. Cyclic [α -³²P]GMP generated during the reaction was purified by a modification [18] of the two-column procedure of Salomon *et al.* [17]. To estimate recovery, 10⁵ c.p.m. of cyclic [8-³H]GMP was routinely added to stopped samples before purification of cyclic GMP. Recoveries were generally 60–70%. Reaction rates for PDE, adenylate cyclase and guanylate cyclase were proportional to added protein and were linear for 15 min when ciliary fraction and substrate were mixed at zero time. Data shown are from single representative experiments which were repeated at least three times.

Free metal-ion concentration was calculated for solutions containing bivalent cations, EGTA and ATP by using the COMICS program [19] extensively modified and translated to BASIC by Stephen Tindall in our laboratory. This program calculates free ion concentrations in complex mixtures of metals and chelators. Stability constants used were from Martell & Smith [20] and Smith & Martell [21].

Sources of chemicals and radioisotopes were as follows: alamethicin, Upjohn (North Haven, CT, U.S.A.); saponin, Calbiochem (San Diego, CA, U.S.A.); W-7 and calmidazolium, Caabco (Houston, TX, U.S.A.); SQ10-009, Squibb (Princeton, NJ, U.S.A.); RO20-1724, Hoffman-LaRoche (Nutley, NJ, U.S.A.); cyclic [2,8-³H]AMP (30 Ci/mmol), cyclic [8-³H]GMP (15 Ci/mmol), [α -³²P]ATP (30–40 Ci/mmol), [α -³²P]GTP (400 Ci/mmol), all from Amersham (Arlington Heights, IL, U.S.A.). All other chemicals were of reagent grade or better.

RESULTS

Adenylate cyclase is present in cilia and in washed cilia (P1)

Cilia prepared by the Ca²⁺-shock deciliation procedure [13] contained readily measurable adenylate cyclase activity. In ten separate experiments the specific activity was 1–5 pmol/min per mg with Mg²⁺ATP as substrate and 25–50 pmol/min per mg with Mn²⁺ATP as substrate. These preparations contained Ca²⁺ carried over from the deciliation procedure, and the addition of 0.1 mM-EGTA to the assay decreased the adenylate cyclase activity with Mg²⁺ATP by at least 5-fold, without influencing the activity with Mn²⁺ATP as substrate (Table 1). When Ca²⁺ and EGTA were added together to buffer the Ca²⁺ concentration in the micromolar range, the inhibition by EGTA was overcome; again the effect was seen only with Mg²⁺ATP as substrate (Table 1). At a higher concentration of Ca²⁺ (0.1 mM), Mg²⁺-adenylate cyclase activity was less than that with micromolar Ca²⁺, and there was no measurable effect on the Mn²⁺-cyclase activity. We consider the activity with Mg²⁺ATP and no Ca²⁺ the 'basal' activity, and the activity with Mn²⁺ATP the 'total' activity, of the adenylate cyclase.

When cilia were osmotically shocked with a 1 mM-Tris/0.1 mM-EDTA solution, about half of their protein was released, but most of the adenylate cyclase activity remained associated with the particulate fraction (called 'P1' hereafter). The adenylate cyclase of P1 had a specific activity similar to that in ciliary preparations (0.8–3 pmol/min per mg) and was also stimulated by Ca²⁺ addition, as described below. Most of the adenylate

Table 1. Ca²⁺ requirement for adenylate cyclase in *Paramecium* cilia

Data are presented as means $\pm$ S.D. of triplicate determinations from a single representative experiment. Cilia were washed in 25 mM-Tris/acetate, pH 7.0, before assay.

Additions	Adenylate cyclase (pmol/min per mg)	
	Mg ²⁺ ATP	Mn ²⁺ ATP
None	5.1 $\pm$ 0.5	49.2 $\pm$ 2.8
0.1 mM-EGTA	0.9 $\pm$ 0.1	48.5 $\pm$ 3.5
1.0 mM-EGTA	1.1 $\pm$ 0.1	33.7 $\pm$ 2.7
0.1 mM-EGTA/0.09 mM-calcium acetate	6.9 $\pm$ 2.4	49.8 $\pm$ 2.7
1.0 mM-EGTA/0.9 mM-calcium acetate	5.0 $\pm$ 1.6	46.8 $\pm$ 2.4
0.1 mM-calcium acetate	2.6 $\pm$ 0.2	48.4 $\pm$ 5.7

cyclase activity of cilia and P1 was latent; treatment of either fraction with membrane-active agents such as Triton X-100 increased the activity about 5-fold.

Cyclic nucleotide phosphodiesterase in cilia and P1: effect on adenylate cyclase assay

To determine whether endogenous cyclic AMP PDE in cilia or P1 influenced the adenylate cyclase assay, we characterized the ciliary PDE. With cyclic AMP as substrate, PDE had a specific activity of 5 nmol/min per mg, which increased 3-fold when cilia or P1 were treated with membrane-permeabilizing agents (results not shown). Much of the PDE of cilia remained associated with ciliary membrane vesicles and was latent. Permeabilizing agents increased the measurable PDE activity by about 5-fold (results not shown).

The dependence of ciliary PDE activity on cyclic AMP concentration yielded a biphasic double-reciprocal plot (results not shown), suggesting the presence of two activities, with K_m values of about 1 μ M and about 5 μ M. These may reflect the separate activities of cyclic AMP PDE and cyclic GMP PDE [22]. The PDE activity in saponin-permeabilized cilia, measured with cyclic AMP as substrate at 0.5 or 20 μ M, was not, however, inhibited by the addition of 200 μ M-cyclic GMP. IBMX, an inhibitor of PDE in other systems, inhibited the PDE of cilia and of ciliary membranes in the millimolar range. The least sensitive PDE was the low- K_m PDE of cilia, which was inhibited 66% and 74% by 1 mM- and 2 mM-IBMX respectively. The PDE activity of ciliary membranes was also inhibited by papaverine (88% inhibition at 2 mM), but was not strongly inhibited by two other PDE inhibitors, RO20-1724 and SQ10-009, at 0.2 mM (results not shown).

The addition of unlabelled cyclic AMP to the adenylate cyclase assay to prevent degradation of labelled product by PDE was without effect at early time points in the cyclase assay, but slightly increased the apparent amount of product at later times. Addition of Ca²⁺ between 0.1 μ M and 0.1 mM had no effect on PDE activity. Interference by PDE activity in the measurement of ciliary adenylate cyclase activity in the presence and absence of Ca²⁺ was therefore negligible.

Adenylate cyclase is regulated by Ca²⁺ concentration

The adenylate cyclase activity of P1 was activated 30-fold by Ca²⁺/EGTA buffers providing free [Ca²⁺] in the micromolar range. Half-maximal activation occurred at 0.88 μ M free Ca²⁺, and at concentrations above 5 μ M free Ca²⁺ inhibition occurred (Fig. 1). The same preparation showed activation of guanylate cyclase with Ca²⁺, but the concentration-dependence was different; half-maximal stimulation required 3.1 μ M free Ca²⁺, and no inhibition was seen up to 10 μ M-Ca²⁺ (Fig. 1). The activation of adenylate cyclase by Ca²⁺ addition was immediate, and was immediately reversed by the addition of excess EGTA (results not shown).

The stimulation of adenylate cyclase induced by Ca²⁺ was the result of an increase in V_{max} , and not of a decrease in K_m for ATP. From the ATP-dependence of the basal and Ca²⁺-stimulated activities (Fig. 2) we calculated K_m values of 83 μ M (basal activity with Mg²⁺-ATP) and 116 μ M (Ca²⁺-stimulated activity); the V_{max} increased from 1.4 to 25.6 pmol/min per mg.

Other bivalent cations were much less effective, or were ineffective, in stimulating the adenylate cyclase. Sr²⁺

Fig. 1. Ca²⁺-dependence of adenylate cyclase (●) and guanylate cyclase (○) activities, assayed on the same P1 fraction

Free Ca²⁺ concentration was varied with a Ca²⁺/EGTA buffer as described in the Experimental section. Arrows indicate the half-maximal activity for both cyclases: for adenylate cyclase and guanylate cyclase 0.88 μ M and 3.1 μ M free Ca²⁺ ion respectively ($K_{0.5}$) was required to achieve this activity. Data are presented as means $\pm$ S.D. of triplicate determinations from a single typical experiment. Assay medium contained 25 mM-Tris/acetate, pH 7.0, 5 mM-magnesium acetate, 40 μ M-ATP or -GTP, 1.5 mM-cyclic AMP or -cyclic GMP, 1 mM-EGTA/Tris, various amounts of CaCl₂, and other assay constituents described in the Experimental section.

Fig. 2. ATP-dependence of P1 adenylate cyclase activity in the presence of Mg²⁺ with (○) or without (□) added Ca²⁺

Data are plotted in a double-reciprocal (Lineweaver-Burk) form, with lines drawn by linear regression. (The point for Mg²⁺ at 20 μ M-ATP was omitted.) Assay conditions used were 5 mM-magnesium acetate, 1 mM-EGTA/Tris, with or without 0.9 mM-calcium acetate and other additions as described in the Experimental section.

Fig. 3. Ion specificity of adenylate cyclase activity assayed in P1 fraction

Free Ca^{2+} (●), Sr^{2+} (○) and Ba^{2+} (▲) concentrations were set by using 1 mM-EGTA buffers. Assay conditions used were 5 mM-magnesium acetate, 1 mM-EGTA/Tris, cations to give the indicated concentrations, and other additions as described in the Experimental section.

stimulated measurably, but only in the range of 0.1 mM, and Ba^{2+} showed no stimulation even up to 0.5 mM (Fig. 3). The EGTA used to control the concentration of free Ca^{2+} was not itself the species that influenced cyclase activity; when free [EGTA] was calculated for these experiments, it was clear that the cyclase activity was not a function of free [EGTA], but of the specific cation present with it. This result also eliminates the possibility that the added bivalent cation indirectly stimulated the enzyme by releasing from EGTA some other trace metal, the true activator of adenylate cyclase.

Although stimulation of adenylate cyclase by Ca^{2+} was consistently observed, the extent of activation was not the same in every preparation. As described below, part of the variability was the result of variable loss of the Ca^{2+} -sensitive component during cell fractionation. In addition, we discovered that the stimulation ratio (activity with Ca^{2+} /basal activity with EGTA) was sensitive to salt concentration and to saponin. The basal activity was more strongly inhibited by NaCl in the assay than was the Ca^{2+} -stimulated activity, so that at 20 mM-NaCl the stimulation ratio was about 100. At 50 mM-NaCl, the adenylate cyclase basal activity was inhibited by 95%. Saponin preferentially inhibited the Ca^{2+} -dependent component of the adenylate cyclase of P1, lowering the stimulation ratio from 20 in the control to 3 in the presence of 1 mg of saponin/ml (Table 2). The temperature of incubation also influenced the extent of stimulation. In one experiment, for example, the stimulation ratio was 12 at 25 °C and only 3.5 at 35 °C.

Inclusion of EGTA in fractionation buffers preserves Ca^{2+} -dependence of adenylate cyclase in sub-ciliary fractions

In their studies of the adenylate cyclase of ciliary membranes, Klumpp and co-workers [23–25] reported that the enzyme was not stimulated by Ca^{2+} , but was inhibited at high Ca^{2+} . When we prepared ciliary membranes as they did, making no special effort to remove the Ca^{2+} left from the deciliation procedure [13],

Table 2. Effect of saponin on basal and Ca^{2+} -dependent adenylate cyclase of P1

Samples of the P1 fraction were preincubated in 25 mM-Tris/acetate, pH 7.0, with or without the addition of saponin for 20 min. The assay was started by the addition of substrate together with other assay constituents described in the Experimental section. Assay medium contained either 1 mM-EGTA or 1 mM-EGTA/0.9 mM-calcium acetate.

Saponin (mg/ml)	Adenylate cyclase (pmol/min per mg)		Stimulation ratio
	Mg^{2+}	$\text{Mg}^{2+} + \text{Ca}^{2+}$	
0	3.4	68.2	20
0.25	3.9	47.7	12
0.5	3.6	27.7	8
0.75	4.6	17.0	3.7
1.0	3.9	12.5	3.2

we also observed that Ca^{2+} did not stimulate membrane adenylate cyclase activity (Table 3). When we prepared ciliary membranes by the same procedure except for the addition of excess EGTA throughout the fractionation, the resulting ciliary membranes contained adenylate cyclase that was stimulated about 15-fold by Ca^{2+} (Table 3) and up to 25-fold in other experiments. The total recovery of adenylate cyclase activity in P1 was improved somewhat by the inclusion of EGTA; P1 contained about the same 'basal' activity (with Mg^{2+} -ATP), and about one-third more 'total' activity (with Mn^{2+} -ATP) when prepared with EGTA. The fraction containing ciliary membrane vesicles (G1) contained similar amounts of basal and total adenylate cyclase whether prepared in the presence or absence of EGTA.

In the same experiment we determined the effect of including EGTA in fractionation buffers on the recovery and distribution of guanylate cyclase (Table 3). With this enzyme, the inclusion of EGTA decreased the recovery both of total guanylate cyclase activity (measured with Mn^{2+}) and of Ca^{2+} -dependent activity (with $\text{Mg}^{2+} + \text{Ca}^{2+}$). Furthermore, the inclusion of EGTA during the fractionation decreased the extent of activation of Ca^{2+} added to the assay, from 34 to 15 in P1, and from 24 to 6 in ciliary membranes (G1) (Table 3). These observations with guanylate cyclase essentially confirm earlier work by Klumpp and co-workers [26–29].

Ca^{2+} -dependent adenylate cyclase is kinetically distinct from Ca^{2+} -dependent guanylate cyclase

Although the two enzymic activities showed different dependences on Ca^{2+} concentration (Fig. 1) and fractionated differently (Table 3) we wanted to eliminate the possibility that the Ca^{2+} -dependent production of cyclic AMP was catalysed by guanylate cyclase, using ATP as an alternative substrate. We therefore examined the effect of ATP on the guanylate cyclase activity (with [^{32}P]GTP as substrate) and the effect of GTP on the adenylate cyclase activity (with [^{32}P]ATP as substrate). Both nucleotides inhibited the respective heterologous cyclases (Fig. 4). The K_m for ATP with the adenylate cyclase was 170 μM , in reasonable agreement with the value of 116 μM obtained in the experiment described in

Table 3. Effect of EGTA in fractionation buffers on recovery of Ca²⁺-dependent adenylate cyclase and guanylate cyclase

Cilia from a single harvest and deciliation were divided into two pools: one fractionated and washed with 0.1 mM-EGTA added (+), the other without (–). Adenylate cyclase and guanylate cyclase activities were then assayed separately in all of the same fractions (see the Experimental section). Ions present in assay were as follows: Mg²⁺, 5 mM-magnesium acetate, 1 mM-EGTA/Tris; Mg²⁺ + Ca²⁺, 5 mM-magnesium acetate, 1 mM-EGTA/Tris, 0.9 mM-calcium acetate (adenylate cyclase) or 0.975 mM-calcium acetate (guanylate cyclase); Mn²⁺, 5 mM-manganese acetate, 1 mM-EGTA/Tris. Data are from a single representative fractionation.

Fraction	EGTA in buffer	Adenylate cyclase (pmol/min per mg)				Guanylate cyclase (pmol/min per mg)			
		Ions in assay			Stimulation ratio	Ions in assay			Stimulation ratio
		Mg ²⁺	Mg ²⁺ + Ca ²⁺	Mn ²⁺		Mg ²⁺	Mg ²⁺ + Ca ²⁺	Mn ²⁺	
Cilia	–	1.2	12.3	47.0	10	3.8	132	38.5	35
	+	1.4	13.8	64.3	9.8	2.2	42.8	18.1	19
P1	–	0.7	5.3	30.6	7.6	3.0	103	42	34
	+	0.9	17.0	41.8	19	2.1	30.8	22.3	15
Sucrose gradient									
G1 (membranes)	–	1.0	1.2	40.4	1.2	6.4	153	67.4	24
	+	1.0	15.0	55.5	15	4.8	30.5	34.3	6.4
G2 (incompletely demembranated cilia)	–	0.4	0.8	20.0	2	2.3	57.9	24.5	25
	+	0.4	7.6	27.7	19	1.4	16.0	12.1	11
G3 (mostly axonemes)	–	0.4	0.4	10.9	1.0	2.3	16.4	6.6	7.1
	+	0.6	1.9	17.1	3.2	0.8	2.7	1.9	3.4

Fig. 4. Effect of GTP on adenylate cyclase (a) and of ATP on guanylate cyclase (b) activities in the P1 fraction

Lineweaver–Burk plots were generated from experiments in which substrate concentration (ATP for adenylate, GTP for guanylate cyclase) was varied in the absence (○) or presence of 0.5 mM (■) or 2.5 mM (●) of the other nucleotide (GTP for adenylate cyclase, ATP for guanylate cyclase). Lines were drawn by linear regression analysis. Assay conditions were 5 mM-magnesium acetate, 1 mM-EGTA/Tris, 0.9 mM-calcium acetate, various concentrations of nucleotides and other conditions as described in the Experimental section.

Fig. 2. GTP inhibition of the adenylate cyclase activity appeared to be competitive, with an apparent K_i of 1.2 mM for GTP (Fig. 4). The K_m for GTP with the guanylate cyclase (46 μ M) was similar to the value of 36–72 μ M obtained by Klumpp & Schultz [27]. ATP

inhibition of the guanylate cyclase activity appeared to be of mixed type. To estimate the K_i for ATP, we used the slope-ratio method [30]. The apparent K_i for ATP was 0.73 mM. Assuming that these calculated K_i values reflect binding of alternative substrates to the catalytic sites, and thus the ability of the guanylate cyclase to catalyse the conversion of ATP into cyclic AMP and of the adenylate cyclase to produce cyclic GMP from GTP, the ‘cross-talk’ between the two enzymes in these crude fractions (cilia, P1) can be estimated and minimized experimentally by choosing appropriate nucleotide concentrations for enzyme assays. On the basis of the K_i , K_m and V_{max} values from Fig. 4, we estimate that, at a concentration of 40 μ M for either nucleotide, more than 90% of the cyclic GMP formation will be catalysed by the guanylate cyclase, and more than 84% of that of cyclic AMP by adenylate cyclase. All of our cyclase assays therefore contained 40 μ M of the appropriate nucleotide to minimize ‘cross-talk’ in the assays.

Is adenylate cyclase a calmodulin-dependent enzyme?

The concentration of Ca²⁺ needed to activate adenylate cyclase (Fig. 1) is in the same range as that needed to activate calmodulin-dependent enzymes [31]. The Ca²⁺-dependent component of adenylate cyclase in cilia was inhibited by the calmodulin antagonists calmidazolium, trifluoperazine and W-7 (Fig. 5). The inhibition was dose-dependent and, at high concentrations of the drugs, complete. Half-maximal inhibition required 5 μ M-calmidazolium, 70 μ M-trifluoperazine or 50–100 μ M-W-7. The response of Ca²⁺-dependent guanylate cyclase to these same drugs was variable. In some experiments no inhibition was observed, whereas in others, such as that shown in Fig. 6, some inhibition occurred. However, inhibition of guanylate cyclase by these calmodulin antagonists never exceeded 50%. At

Fig. 5. Effect of calmodulin-antagonist drugs on P1 adenylate cyclase activity

Calmidazolium (●), W-7 (○) or trifluoperazine (■) was added at different concentrations to assay medium containing 5 mM-magnesium acetate, 1 mM-EGTA/Tris, 0.9 mM-calcium acetate and other additions described in the Experimental section. Control activity in the presence of 1% ethanol carrier was 24 pmol/min per mg.

Fig. 6. Effect of calmodulin-antagonist drugs on P1 guanylate cyclase activity

Calmidazolium (●), W-7 (○) or trifluoperazine (■) was added at different concentrations to assay medium as described in Fig. 4, except that guanine nucleotides were present and the Ca^{2+} /EGTA ratio was 0.975. Control activity was 64 pmol/min per mg.

high concentrations of calmidazolium (35–100 μM), a large stimulation of guanylate cyclase was consistently observed. At the concentration of calmidazolium that stimulated guanylate cyclase 4-fold, adenylate cyclase was completely inhibited (cf. Figs. 5 and 6).

In several experiments we attempted, without success, to reconstitute Ca^{2+} -dependent adenylate cyclase in P1 or ciliary-membrane vesicles prepared in the absence of EGTA by the addition of purified *Paramecium* calmodulin (generously given by R. Hinrichsen, University

of Wisconsin–Madison). The addition of *Paramecium* calmodulin to EGTA-washed ciliary-membrane vesicles or to P1 stimulated Ca^{2+} -activated guanylate cyclase, raising the specific activity from 60 to 102 pmol/min per mg in one case, and from 205 to 320 in another.

The reason for the loss of Ca^{2+} -sensitivity by adenylate cyclase when EGTA is omitted is unknown. When the soluble material released from cilia by washes not containing EGTA was concentrated by ultrafiltration (Amicon PM-10 membrane with cut-off of 10 kDa) and added back to the Ca^{2+} -insensitive adenylate cyclase preparations, there was no restoration of Ca^{2+} -sensitivity (results not shown).

DISCUSSION

In *Paramecium tetraurelia*, both Ca^{2+} and cyclic nucleotides are believed to regulate the beat of the several thousand cilia that cover the cell's surface and propel it through its surrounding medium [4–9]. Membrane depolarization induced by mechanical, chemical or thermal stimuli causes Ca^{2+} channels in the ciliary membrane to open, allowing extracellular Ca^{2+} ion to enter the ciliary space, where it triggers a reversal in the direction of the ciliary power stroke [1–5]. We [7–9] and others [6,32] have recently reported that cyclic nucleotides also regulate ciliary motion in *Paramecium*. Conditions that lead to elevated cyclic AMP concentrations in living cells bring about faster ciliary beating, and, in permeabilized 'models' re-activated with ATP, the addition of cyclic AMP increases swimming speed by altering the orientation of the ciliary power stroke, and probably its beat frequency as well [5,6,8,9]. Direct micro-injection of cyclic AMP into the cytoplasm of *Paramecium* results in membrane hyperpolarization and increased ciliary beat frequency [7].

Our discovery that the adenylate cyclase of cilia is strongly stimulated by sub-micromolar concentrations of Ca^{2+} and inhibited by Ca^{2+} concentrations above micromolar suggests a functional interplay between Ca^{2+} and cyclic AMP in ciliary regulation. Satir [33] has reviewed the literature on regulation of the ciliary and flagellar beat, and has proposed a 'two-switch' model in which Ca^{2+} and cyclic nucleotides act at separate switch points that determine which of the nine outer-doublet microtubules will slide actively at a given instant during the cycle of a single ciliary (flagellar) beat. To explain the complex three-dimensional ciliary stroke, one has to postulate both temporal (within milliseconds) and spatial (within the cilium) gradients of the putative regulators (Ca^{2+} and cyclic nucleotide). Superimposed on the events that govern a normal stroke of the cilium, there must be mechanisms that mediate the alterations of beat frequency and direction that occur in response to external stimuli. Clearly, co-ordinated regulation of Ca^{2+} and cyclic AMP concentrations is essential if they are co-regulators of the ciliary beat.

The concentration of ciliary Ca^{2+} is set by Ca^{2+} influx through voltage-gated Ca^{2+} channels located uniquely in the ciliary membrane, and by Ca^{2+} efflux [34,35] or sequestration by uncharacterized mechanisms. The steady-state concentration is believed to be below 0.1 μM , but, after a depolarizing stimulus, intraciliary [Ca^{2+}] probably exceeds micromolar [1]. It is not known

whether the Ca²⁺ channels are randomly distributed about the ciliary membrane surface or are localized. The steady-state concentration of ciliary cyclic AMP is presumably set by the balance of adenylate cyclase and cyclic AMP PDE activity; there is no measurable release of cyclic AMP from cells [8]. Our studies of the ciliary PDE(s) suggest no regulation of this activity by Ca²⁺; the same conclusion was reached by von Hirschhausen [22]. (Kudo *et al.* [36] also reported that the PDEs of the ciliate *Tetrahymena* were not activated by Ca²⁺ plus calmodulin.) Given the 20-fold activation of ciliary adenylate cyclase by Ca²⁺ that we have documented here, it seems likely that depolarization and the consequent increase in ciliary Ca²⁺ will result in a transient increase in ciliary cyclic AMP, and some alteration of the ciliary beat [6–9].

We [8] and Schultz *et al.* [37] have described fluctuations in total cell cyclic AMP concentrations in response to the addition of various ions that depolarize or hyperpolarize the membrane and alter the swimming behaviour of *Paramecium*. We observed a decrease in total cell cyclic AMP after depolarization with external K⁺ [8]; the same response was seen in two mutants which differ from wild type in the amount of Ca²⁺ entry that accompanies depolarization (pawn, which shows less Ca²⁺ entry, and dancer, which shows more; M. C. Gustin, unpublished work). Hyperpolarizing conditions (low external K⁺), which tend to keep ciliary Ca²⁺ channels closed, resulted in a sharp rise in total cell cyclic AMP [8]. Thus our evidence *in vitro* for regulation of cyclic AMP by Ca²⁺ (the present paper) and the measurements *in vivo* of cyclic AMP under conditions where intraciliary Ca²⁺ presumably varies widely appear contradictory. It is not clear how much the ciliary compartment contributes to the total cyclic AMP content of a cell, but it seems likely that much of the observed change in cyclic AMP occurs in cell bodies, not in cilia. Immunocytochemical studies suggest an equal concentration of cyclic AMP in bodies and cilia [38], and cilia represent a small fraction of the cell volume. Perhaps changes in total cell cyclic AMP mask local changes in cilia, or perhaps the inhibition of adenylate cyclase at high Ca²⁺, which we observe *in vitro*, also occurs *in vivo*, complicating the effects of Ca²⁺ influx after strongly depolarizing stimuli.

The ciliary membrane of *Paramecium* also contains a guanylate cyclase, first described by Schultz & Klumpp [26] and shown by them to be stimulated by Ca²⁺, apparently via calmodulin [27–29]. They established that the adenylate cyclase and the guanylate cyclase of cilia were distinct entities which could be physically separated [24]. We compared the two cyclase activities and showed by several criteria that Ca²⁺-regulated adenylate cyclase is not merely the activity of a guanylate cyclase acting on ATP instead of GTP. The two Ca²⁺-regulated cyclases have different sensitivities to Ca²⁺ (Fig. 1) and to calmodulin antagonists (Fig. 5). Washing the ciliary membrane with EGTA-containing solutions resulted in an increase in Ca²⁺-activated adenylate cyclase activity, while Ca²⁺-activated guanylate cyclase activity decreased (Table 3). Finally, Ca²⁺-activated guanylate cyclase, but not adenylate cyclase, could be stimulated by added calmodulin. Schultz and co-workers [25] have described large increases in total cell cyclic GMP content in response to depolarization. Since either cyclic AMP or cyclic GMP can alter ciliary motion in permeable 'models' ([6,8,32]; N. M. Bonini & D. L.

Nelson, unpublished work), the existence of the Ca²⁺-dependent guanylate cyclase in cilia poses a further complication in the interpretation of cyclic nucleotide fluctuations *in vivo* and their physiological significance.

Our characterization of the *Paramecium* adenylate cyclase suggests close analogy between this ciliate enzyme and the Ca²⁺-dependent adenylate cyclases of brain, sperm, and a variety of other tissues of multicellular organisms (reviewed in [39–42]). The adenylate cyclase that we have studied is located in an electrically excitable membrane that regulates the motility of an axoneme; we were therefore particularly interested in comparing it with the adenylate cyclases of nervous tissue and of sperm. Like the enzyme from these sources, the *Paramecium* adenylate cyclase is activated in the range of 0.1–1 μ M (free) Ca²⁺, and inhibited by concentrations above 5 μ M; it also shares with the sperm enzymes their strong activation by Mn²⁺ and their insensitivity to fluoride, forskolin and guanine nucleotides ([23]; M. C. Gustin, unpublished work). Although this insensitivity to agents that influence the hormone-sensitive cyclases was considered evidence that the catalytic activities of the Ca²⁺-sensitive cyclases did not associate with guanine-nucleotide-binding proteins, several recent studies suggest that the two types of cyclases may not be fundamentally different. Kopf *et al.* [43] showed that in abalone sperm, in which the Ca²⁺-activated adenylate cyclase had been previously shown to be insensitive to guanine nucleotides and to ADP-ribosylation by cholera or pertussis toxin [44,45], there is a protein that can be ADP-ribosylated by pertussis toxin under some conditions, and which co-migrates with the α 1-subunit of the inhibitory guanine-nucleotide-binding protein from another source. Further, antibodies against the 35 kDa β -subunit of the guanine-nucleotide-binding protein reacted with a protein of that size from the abalone sperm. Eisenschlos *et al.* [46] also showed that the Ca²⁺-dependent adenylate cyclase of *Trypanosoma*, although not itself sensitive to guanine nucleotides, came under the control of guanine nucleotides when reconstituted with regulatory proteins from liver. If the similarities between the *Paramecium* enzyme and the other Ca²⁺-sensitive cyclases extend to this property, one might predict the existence of guanine-nucleotide-binding proteins in *Paramecium*.

The activation of the cyclases from brain and sperm by low concentrations of Ca²⁺ [39,40] is apparently mediated by calmodulin, reversibly associated with the catalytic subunit. A variety of calmodulin antagonists, including trifluoperazine, fluphenazine and W-7, prevent the stimulation of these cyclases by Ca²⁺, and in some cases it has been possible to resolve catalytic activity from calmodulin (losing Ca²⁺-sensitivity) and to reconstitute the Ca²⁺-dependence by addition of purified calmodulin. We also observed a strong inhibition of the *Paramecium* cyclase by calmodulin antagonists, at concentrations slightly higher than those needed to inhibit other calmodulin-dependent enzymes, including adenylate cyclases. However, inhibition by these drugs cannot be considered unambiguous evidence of calmodulin's involvement; they inhibit several enzymes that clearly do not depend on calmodulin [47], including a Ca²⁺-ATPase from *Paramecium* (A. L. Levin, S. M. Travis, L. D. DeVito and D. L. Nelson, unpublished work), and they do not fully inhibit the Ca²⁺-dependent guanylate cyclase of *Paramecium* (Fig. 6),

believed on other grounds to be a calmodulin-dependent enzyme [27–29].

We were unable to demonstrate reconstitution of Ca^{2+} -dependent adenylate cyclase activity by the addition of purified calmodulin from *Paramecium*, but we did observe a striking loss of Ca^{2+} -sensitivity without concomitant loss of 'basal' activity under several circumstances. When cilia or sub-ciliary fractions were prepared in the presence of Ca^{2+} , the adenylate cyclase activity was only slightly affected by EGTA or Ca^{2+} added to the assay. When we assayed the enzyme at higher temperature (35 °C instead of the normal 25 °C) the stimulation by Ca^{2+} was less striking. The Ca^{2+} stimulation ratio was also decreased (from 20 to 3) by the addition of the membrane-active agent saponin to the assay, further suggesting that some factor that conferred Ca^{2+} -sensitivity on the adenylate cyclase was labile. Schultz & Klumpp [23], who first described the ciliary adenylate cyclase of *Paramecium*, reported that their enzyme (prepared in the presence of Ca^{2+} and assayed at 37 °C) showed no stimulation by Ca^{2+} and no inhibition by 100 μM - La^{3+} or EGTA. We confirmed this observation, and showed in side-by-side experiments that the inclusion of EGTA during the preparation of cilia was the factor that accounted for the difference between the earlier results of Schultz & Klumpp [23] and our results.

We do not know why Ca^{2+} -sensitivity is lost during fractionation in Ca^{2+} , or during treatment with saponin. It is possible that calmodulin or some other subunit of the cyclase dissociates in high Ca^{2+} , but not in EGTA; this is certainly not the usual pattern with calmodulin, but we have detected at least one calmodulin-binding protein in cilia that binds radiolabelled calmodulin only in the presence of EGTA (T. C. Evans & D. L. Nelson, unpublished work). Alternatively, a Ca^{2+} -stimulated proteinase or lipase might perturb the membrane environment of the cyclase so as to destroy the Ca^{2+} -dependence, as saponin apparently does. The recent isolation of mutants of *Paramecium* with apparently defective calmodulin [48] affords the opportunity to determine definitively whether the adenylate cyclase of *Paramecium* is stimulated by Ca^{2+} via calmodulin.

Most of the adenylate cyclases that have been characterized are from vertebrates or higher invertebrates; less is known about the enzyme in simple eukaryotes. Watanabe & Nozawa [49] found no adenylate cyclase associated with the cilia of *Tetrahymena pyriformis*, but showed that an activity associated primarily with pellicles (surface membranes) was inhibited by high (0.2 mM) Ca^{2+} concentrations. Calmodulin addition to this preparation did not affect its activity. Nagao *et al.* [50,51] reported that the particulate adenylate cyclase of *Tetrahymena* was inhibited by trifluoperazine or W-7, an effect they attributed to non-specific action on the membrane, not to a calmodulin/cyclase interaction. Kudo *et al.* [52] reported that the adenylate cyclase of the *Tetrahymena* plasma membrane was insensitive to fluoride, forskolin or guanine nucleotides, and was inhibited by Ca^{2+} in the micromolar range. They did not detect a requirement for or stimulation by submicromolar Ca^{2+} .

Torres and co-workers have purified and characterized adenylate cyclase from *Trypanosoma cruzi* [53]. In this protozoan, the enzyme is inhibited by high Ca^{2+} , but not stimulated by Ca^{2+} in the range studied [54]. The purified

catalytic subunit cross-reacts immunologically with enzymes from *Neurospora* and *Mucor*, but not with the vertebrate adenylate cyclase [53]. As noted above reconstitution of the *Trypanosoma* cyclase with regulatory factors from liver membranes confers sensitivity to guanine nucleotides [46], suggesting some fundamental homology between the trypanosomal and vertebrate enzymes. The soluble adenylate cyclase from *Neurospora crassa* could be activated by calmodulin, and EGTA reversed this activation [55]. The *Neurospora* enzyme was not stimulated by fluoride, guanine nucleotides or cholera toxin [56]. It seems likely that further comparisons of the metazoan and protozoan adenylate cyclases will shed light on the evolution of the hormone-sensitive mechanisms of the metazoans.

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