Activation of Adenylyl Cyclase in *Chlamydomonas reinhardtii* by Adhesion and by Heat

Tatsuaki Saito, Linda Small, and Ursula W. Goodenough

Biology Department, Washington University, St. Louis, Missouri 63130

Abstract. Adhesion between *Chlamydomonas reinhardtii* gametes generates a rapid rise in cAMP levels which stimulates mating responses and zygotic cell fusion (Pasquale and Goodenough, 1987). We show here that sexual adhesion in vivo results in a twofold stimulation of flagellar adenylyl cyclase activity when the enzyme is subsequently assayed in vitro, a stimulation that is specifically blocked by Cd^{2+}. A twofold stimulation is also elicited by the in vitro presentation of soluble cross-linking reagents (antisera and concanavalin A). In contrast, the 10–30-fold stimulation of the flagellar cyclase by in vitro exposure to 40°C, first described by Zhang et al. (1991), is insensitive to Cd^{2+} but sensitive to such drugs as trifluoperizine and dibucaine. The capacity for twofold stimulation is displayed by the vegetative and gametic enzymes but is lost when gametes fuse to form zygotes; in contrast, the 10-fold stimulation is displayed by the gametic and zygotic enzymes but not the vegetative enzyme. The signal-defective mutant *imp-3* fails to generate the normal mating-triggered cAMP production and can be rescued by exogenous dibutyryl cAMP. It displays normal basal rates of flagellar cyclase activity and a normal twofold stimulation by sexual adhesion and by soluble cross-linkers, but it is defective in 40°C activation. The gametic cell-body adenylyl cyclase is stimulated when wild-type flagella, but not *imp-3* flagella, undergo adhesive interactions in vivo, and it can be directly stimulated in vitro by cAMP presentation. We propose that the two levels of flagellar cyclase stimulation reflect either sequential steps in the activation of a single cyclase enzyme, with *imp-3* blocked in the second step, or else the sequential activation of two different flagellar enzymes, with *imp-3* defective in the second enzyme. We further propose that the cell-body enzyme is activated by the cAMP that is generated when flagellar cyclase activity is fully stimulated.

THE *Chlamydomonas* life cycle includes four cell types (see Harris, 1989). (*a*) Vegetative cells are haploid, and mitotic; (*b*) gametic cells differentiate from nitrogen-starved vegetative cells. They are of two mating types (mt)[1] plus (mt^+) and minus (mt^-). When mixed together, they agglutinate via mt-specific flagellar agglutinins and quickly fuse in pairs. (*c*) Zygotic cells result from gametic fusion. Their nuclei fuse ~1 h after cell fusion and they initiate dormant spore formation ~2 h later. (*d*) Zygospores are thick-walled mature spores which, with appropriate stimulation, undergo meiosis and release haploid vegetative cells, two mt^+ and two mt^-.

The gametic mating reaction uses a signal-transduction cascade. Agglutination stimulates a rise in intracellular cAMP (Pijst et al., 1984; Pasquale and Goodenough, 1987; Kooijman et al., 1990); the elevated cAMP stimulates such mating responses as the secretion of a wall-degrading enzyme called GLE (Kinoshita et al., 1992), loss of the cell wall, actin polymerization, and cell fusion (for review see Goodenough, 1991). These mating responses can also be elicited in gametes of a single mt by the exogenous presentation of dibutyryl cAMP (db-cAMP) (Pasquale and Goodenough, 1987; Goodenough, 1989, 1993; Hunnicutt and Snell, 1990; Kooijman et al., 1990; Goodenough et al., 1993). Several drugs have been identified which block the adhesion-induced cAMP elevation and mating responses and are referred to as upstream inhibitors (Pasquale and Goodenough, 1987; Goodenough et al., 1993). The protein kinase inhibitors H-8 and staurosporine are downstream inhibitors in that they permit cAMP elevation in vivo but prevent mating responses, presumably via their ability to block protein phosphorylation.

The *Chlamydomonas* flagellar adenylyl cyclase gives no evidence of G-protein modulation (Pasquale and Goodenough, 1987; Zhang et al., 1991), but various observations suggest that it might be regulated by Ca^{2+} (Goodenough et al., 1993). The flagellum contains both calmodulin (Gitelman and Witman, 1980) and centrin (Huang et al., 1988*a*; Piperno et al., 1992; J. Salisbury, personal communication), a calmodulin-related protein (Huang et al., 1988*b*). Therefore, the Ca^{2+} effects may well be mediated by calmodulin-like proteins, an inference supported by the fact that several

1. *Abbreviations used in this paper*: ConA, concanavalin A; db, dibutyryl; GLE, gametic lytic enzyme; IBMX, isobutylmethyl xanthine; mt, mating type.

upstream inhibitors are calmodulin-binding drugs such as TFP and W-7 (Detmers and Condeelis, 1986; Pasquale and Goodenough, 1987; Goodenough et al., 1993).

The present report describes studies on the vegetative, gametic, and zygotic flagellar adenylyl cyclase and the gametic cell-body adenylyl cyclase from wild-type *C. reinhardtii* and from the signal-defective mutant strain *imp*-3 (Goodenough et al., 1976). We show that flagellar enzyme activity can be stably stimulated twofold either by the native adhesion reaction or by soluble ligands that bind to the flagellar membrane, and at least 10-fold by 40°C incubation in vitro (Zhang et al., 1991). These two modes of activation differ in their life-cycle expression, in their sensitivity to various drugs, and in their manifestation in the *imp-3* mutant, leading us to propose that the twofold stimulation reflects a stable sensitivity to cross-linking that is lost with zygote formation, whereas the 10-fold stimulation reflects a labile responsiveness to sexual adhesion that is specific for gametes.

This is the first report of an adenylyl cyclase activated by adhesion, and the first report of a *Chlamydomonas* mutant strain specifically blocked in the gametic signal transduction pathway.

Materials and Methods

Strains and Cell Culture

Wild-type strains (CC-620 and CC-621, *Chlamydomonas* Genetics Center, Duke University, Durham, NC) were used in most experiments. Some experiments used the mutant strains *imp-1* mt^+ (CC-1158) (Goodenough et al., 1982), *imp-3* mt^+ (CC-465), *imp-3* mt^- (CC-466) (Goodenough et al., 1976), *imp-5* mt^+ (CC-469) (Adair et al., 1983), *imp-10* mt^- (CC-1147) (Hwang et al., 1981), and *bald-2* mt^+ (CC-478) (Goodenough and St. Clair, 1975). Vegetative cells were grown in liquid Tris-acetate-phosphate (TAP) medium (Gorman and Levine, 1965) for 3 d under constant illumination. Gametes were obtained from plate culture (Martin and Goodenough, 1975): cells were grown for 1 wk on 1.5% agar plates with TAP medium; they were flooded in nitrogen-free medium (Matsuda et al., 1971), washed with the same medium, and incubated for 2–3 h. Mating efficiency was determined by counting biflagellate (BF) and quadriflagellate (QF) cells in glutaraldehyde-fixed samples using phase-contrast microscopy and applying the formula: % mating = 2QF × 100/(2QF + BF). Cell-wall loss was determined by exposing a known number of cells to 0.1% NP-40, which lyses wall-free cells, and counting the remaining intact cells with a hemacytometer.

cAMP Levels In Vivo

cAMP levels in vivo were determined by radioimmunoassay as described by Pasquale and Goodenough (1987). Each sample was assayed in triplicate.

Preparation of Gametic Lytic Enzyme

Walled gametes of each mt were mixed together. After a 15-min incubation, cells were pelleted by centrifugation at 13,000 *g* for 10 min. Flagellar membrane vesicles shed during mating were removed by centrifugation at 100 K × *g* for 1 h, and the supernatant was used as a crude gametic lytic enzyme (GLE) fraction.

Preparation of Cell Bodies and Flagella during Mating

Gametes were treated with GLE to remove their cell walls and resuspended in nitrogen-free medium at 1 × 10^8 cells/ml. For cell-body preparations, each sample was freeze thawed in dry ice/propanol at indicated times, a procedure that both detaches flagella from cell bodies and ruptures the cell bodies. Thawed samples were centrifuged at 4,000 *g* for 5 min, and the pellet (containing ruptured cell bodies but not flagella) was resuspended in 10 mM Tris-HCl, pH 7.5, 1 mM DTT and 5 mM $MgSO_4$ (buffer A). For flagellar preparations, flagella were isolated at indicated times by the dibucaine method (Pfister et al., 1982), mixed with sucrose to a final concentration of 4%, overlayed onto 30% sucrose, and spun at 400 *g* for 5 min. The upper layer was collected and centrifugated at 13,000 *g* for 10 min. The pellet was resuspended in buffer A and freeze thawed (determined to be necessary to permeabilize the flagellar membranes to ATP). Protein content was determined by the Bio-Rad protein assay (BioRad Labs., Hercules, CA).

Preparation of Cell Bodies and Flagella for In Vitro Experiments

For cell-body preparations, gametes were treated with GLE and resuspended in nitrogen-free medium at 1 × 10^8 cells/ml. Cells were freeze thawed and centrifuged at 400 *g* for 5 min to remove the flagella. The pellet was resuspended in an equal volume of nitrogen-free medium and treated with drugs, soluble cross-linkers, or heat. The sample was recentrifuged at 400 *g* for 5 min and resuspended in equal volume of buffer A. For flagellar preparations, flagella were detached from walled gametes (1 × 10^8 cells/ml) by pH shock (Witman et al., 1972), mixed with sucrose to a final concentration of 4%, and spun at 400 *g* for 5 min to remove the cell bodies. The supernatant (1.5 ml) was freeze thawed to permeabilize the flagellar membranes and then treated with drugs, soluble cross-linkers, or heat. After treatment, flagella were harvested by centrifugation at 13,000 *g* for 10 min and resuspended in 200 μl of buffer A.

Adenylyl Cyclase Assay

Adenylyl cyclase activity in cell-body and flagellar preparations was essentially assayed according to the method of Mittal (1986). The reaction mixture (0.1 ml) contained 30 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4$, 0.5 mM ATP, 0.2 mM isobutylmethyl xanthine (IBMX), 1 mM DTT, 7.5 mM creatine phosphate, and 15 U/ml creatine phosphokinase. α [^{32}P]ATP (sp act 30 Ci/mmol; New England Nuclear, Boston, MA) was present at 2–5 × 10^6 cpm/sample. Reactions were initiated by adding sample to the prewarmed reaction mixture and incubated for 10 min at 30°C; at this time point, reaction rates remain linear (see Zhang et al., 1991) (data not shown). The reaction was terminated by adding 25 μl of 30% TCA. To each assay, 1–2 × 10^5 cpm cyclic [^{3}H]AMP was added as recovery marker, and insoluble debris was removed by centrifugation at 13,000 *g* for 10 min. The isolation of labeled cyclic AMP was achieved by the method of Salomon et al. (1979). Each sample was assayed in triplicate. Rates are expressed as specific activities except for experiments where exogenous protein is added (antiserum, concanavalin A), in which case rates are expressed on a per-flagellum basis (as determined by cell counts). All experiments were performed at least twice with comparable results; most were performed 3–4 times with comparable results. Except where otherwise noted, data are results from a single representative experiment.

Chemicals

Sodium vanadate was purchased from Fischer Scientific Co. (Fair Lawn, NJ) and concanavalin A (Con A) from E-Y Lab Co. (San Mateo, CA). The other reagents were purchased from Sigma Immunochemicals (St. Louis, MO). The inhibitors were suspended in deionized water except staurosporine and lidocaine in ethanol; appropriate ethanol controls were performed. Inhibitors were used at the following final concentrations: $LaCl_3$, 100 μM; $CdCl_2$, 100 μM; staurosporine, 1 μM; TFP, 30 μM; lidocaine, 1.25 mM; dibucaine, 2.5 mM; diltiazem, 125 μM; EGTA, 2 mM; nucleotides, 1 mM; sodium vanadate, 100 μM. As documented in Goodenough et al. (1993) and Goodenough (1993), none of these inhibitors is toxic to the cells at the concentrations used, and their effects can be reversed by washing.

Results

In Vivo cAMP Responses in Wild-type and Mutant Strains

Fig. 1 shows the normal kinetics of gametic cell fusion (Fig. 1 *A*) and cAMP production (Fig. 1 *B*) in a wild-type mating (○). Levels of cAMP spike as soon as adhesion is underway, and fall when the gametes fuse and the flagella disadhere (Pasquale and Goodenough, 1987).

Gametes of the same mt can also be induced to undergo adhesion by incubating them in the presence of a polyclonal antiserum (α-fla) raised against gametic flagella; the resultant isoagglutination elicits all known mating responses, ex-

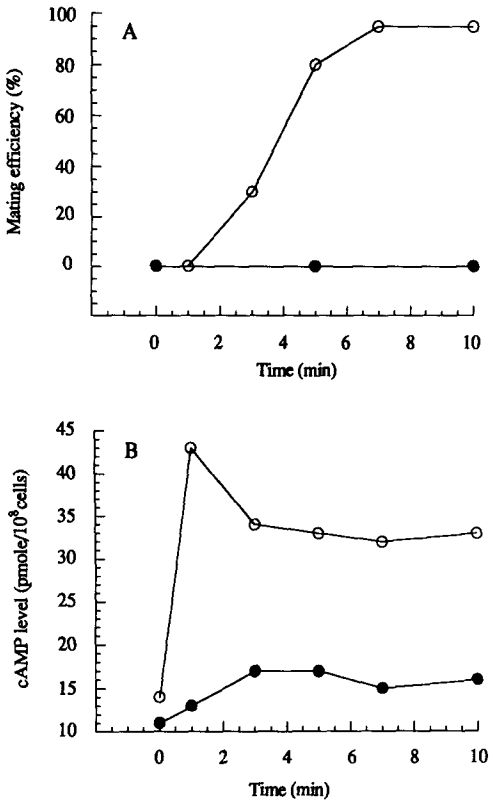

Figure 1. Time course of mating responses and cAMP elevation in wild-type and *imp-3* matings. (*A*) Mating efficiency; (*B*) cAMP levels. Wild-type gametes (○); *imp-3* gametes (●).

cept cell fusion, in both wild-type and agglutinin-defective mutant strains (Goodenough and Jurivich, 1978). Table I documents that, as expected, α-fla isoagglutination also induces elevated cAMP levels in both wild-type *mt*⁺ gametes and in gametes of the agglutinin-defective *imp-5 mt*⁺ mutant strain.

The mutant strain *imp-3*, in contrast, is defective in the production of cAMP in response to adhesion. The original *imp-3 mt*⁺ mutant was described (Goodenough et al., 1976) as displaying strong agglutination with wild-type *mt*⁻ strains but fusing with poor and variable efficiency. Genetic analysis showed it to be unlinked to mt, allowing the recovery of *imp-3 mt*⁻ meiotic products (Goodenough et al., 1976). In a recent re-analysis of the *imp-3* mutation, *imp-3 mt*⁺ × *imp-3 mt*⁻ matings were performed. Agglutination was again found to be strong, but no mating responses ensued: there was no wall loss and no cell fusion (Fig. 1 *A*, •) even if the walls were first removed with GLE. Moreover, the initial strong adhesion soon abated, indicating a failure to recruit new agglutinins to replace agglutinins inactivated by adhesion (Snell and Roseman, 1979; Snell and Moore, 1980), a cAMP-dependent event (Goodenough, 1989; Hunnicutt et al., 1990). When 10 mM db-cAMP/1 mM IBMX was provided to such disadhered cells, agglutination soon resumed, walls were lost, the cells fused, and normal zygote development ensued. Similarly, *imp-3* gametes of one mt were observed to undergo normal wall loss when provided with db-cAMP/IBMX. Hence the *imp-3* mutants behave as if they are blocked in upstream cAMP generation and not downstream responsiveness to cAMP.

Direct documentation for this inference is given in Fig. 1 and Table I. Fig. 1 *B* (•) shows that during an *imp-3* × *imp-3* mating, only a modest increase in cAMP levels takes place. Moreover, the isoagglutination of *imp-3 mt*⁺ gametes with α-fla fails to stimulate cAMP generation (Table I).

The foregoing in vivo observations document a positive correlation between adhesion, cAMP production, and mating responses in normal gametes, and a defect in this pathway incurred by the *imp-3* mutation. The most straightforward explanation for such a correlation is that adhesion activates adenylyl cyclase activity in the wild type but not in the mutant. However, the same outcome would occur if, for example, adhesion instead inhibited a cAMP phosphodiesterase. We have therefore undertaken an experimental analysis of adenylyl cyclase activity in flagellar and in cell-body preparations from the wild-type and the *imp-3* mutant strains. To simplify presentation of the data, it is assumed that only one type of adenylyl cyclase is present in the flagellum and that a second type is present in the cell body. This assumption is evaluated in the Discussion.

Basal Adenylyl Cyclase Rates and Specific Activities

Flagellar cyclase activity was analyzed in two types of preparations: for in vivo time-course studies, where rapid deflagellation is important, flagella were released from the cells using dibucaine, whereas for in vitro studies it is necessary to use pH shock because dibucaine inhibits a facet of the in vitro response, as documented below. In both cases, the harvested flagella were subsequently made permeable to ATP by freeze thawing. Cell-body cyclase activity was analyzed in GLE-treated gametes that were freeze thawed to both permeabilize their membranes and release their flagella.

Table II presents basal adenylyl cyclase activities for each type of flagellar preparation from wild-type and *imp-3* ga-

Table I. Effect of α-fla Antiserum on Cell Wall Loss and cAMP Levels in Wild-type and Mutant Strains

Strain	Percent of cell wall-less cells		cAMP level	
	−Antiserum	+Antiserum	−Antiserum	+Antiserum
			pmole/10⁸ cells	
Wild-type *mt*⁺ gametes	0	75	12	80
imp-5 mt⁺ gametes	0	95	12	70
imp-3 mt⁺ gametes	0	0	14	17
Wild-type vegetative *mt*+	0	0	23	28

Gametic cells (1 × 10⁸ cells/ml) were mixed with 0.3 mg/ml α-fla antiserum and incubated for 10 min; for vegetative cells, antiserum was at 0.1 mg/ml.

Table II. Basal Adenylyl Cyclase Rates

Source	Specific activity			Total activity		
	pH shock	Dibucaine	Freeze-thaw	pH shock	Dibucaine	Freeze-thaw
	pmole/mg/min			*pmole/10^8 cells/min*		
Gametic flagella (wt)	54.0 ± 3.4	19.6 ± 1.7		1.2 ± 0.2	0.5 ± 0.04	
imp-3 gametic flagella	58.3 ± 10.7	13.5 ± 1.2		1.3 ± 0.2	0.3 ± 0.04	
Zygotic flagella (1 h) (wt)	49.1 ± 8.4	20.4 ± 0.1		0.9 ± 0.1	0.6 ± 0.1	
Vegetative flagella (wt)	40.5 ± 4.0	41.0 ± 2.9		0.8 ± 0.1	0.8 ± 0.1	
Gametic cell body (wt)	23.4 ± 2.0	22.9 ± 1.9	24.1 ± 2.0	10.1 ± 0.6	8.4 ± 0.6	8.8 ± 0.8
imp-3 gametic cell body			33.8 ± 2.3			12.6 ± 0.9

Basal rates were assayed on preparations maintained on ice from the time of deflagellation and warmed to 30°C at the time of assay. Each rate is the average of at least three experiments.

metes, as well as from wild-type vegetative cells and zygotes. The basal rates for dibucaine-released flagella are consistently lower than for pH-shock flagella in gametic and zygotic preparations, whereas they are comparable in vegetative preparations.

The cyclase associated with pH-shock gametic flagella is quantitatively pelleted by a 15-min centrifugation at 30,000 *g*, indicating that it is not released by freeze thawing and is presumably membrane associated. Treatment of these flagella with the detergent lubrol releases most of the activity into the 30-kD supernatant, and the detergent-soluble activity is comparable to the membrane-associated activity (Table III, column 1).

Also shown in Table II are basal rates for the cell-body adenylyl cyclase. The specific activity of the cell-body enzyme is less than half that of the flagellar enzyme (Table II, columns 1–3) but it in fact accounts for most (>90%) of the cyclase activity on a per-cell basis (Table II, columns 4–6), as also reported for *C. eugametos* (Kooijman et al., 1990). Rates for the cell-body enzyme are comparable in gametes exposed to pH shock or dibucaine (Table II), so the dibucaine-induced depression of basal activity is restricted to the gametic flagellar enzyme.

Response of Adenylyl Cyclase to In Vivo Adhesion

To test the postulate that sexual adhesion directly stimulates adenylyl cyclase activity, we isolated flagella from unmated gametes and gametes harvested at various stages of the mating reaction, separated flagella from cell bodies, and assayed each for adenylyl cyclase activity in vitro. The results are shown in Fig. 2. Flagellar cyclase increases immediately after the gametes are mixed, peaks at the onset of cell fusion, and plateaus at an intermediate position (Fig. 2 *B*, ○). The cell-body enzyme also increases activity but shows a lag in responsiveness, peaking after the flagellar enzyme is maximally stimulated (Fig. 2 *C*, ○).

Table III. Effect of Lubrol on Activation of Flagellar Adenylyl Cyclase

	Flagellar adenylyl cyclase activity		
	No treatment	+α-fla antiserum	+40°C
	pmole/2 × 10^8 flagella/min		
No addition*	1.3	2.1	8.6
Lubrol addition*	1.3	2.0	0.4
Lubrol extract‡ (30,000 *g* sup)	0.9	1.1	0.6

* Freeze-thawed *mt+* flagella (2 × 108 flagella/ml) were treated with α-fla antiserum (0.17 mg/ml) or heat (40°C) for 15 min, harvested by centrifugation at 13,000 *g* for 10 min, and resuspended in buffer A or buffer A containing 0.1% lubrol. Each sample was incubated for 15 min on ice and assayed.
‡ Freeze-thawed *mt+* flagella (2 × 10^8 flagella/ml) were extracted with 10 mM Tris-HCl, pH 7.5, and 0.1% lubrol for 15 min on ice and the insoluble portion was removed by centrifugation at 30,000 *g* for 15 min. The supernatant was treated with α-fla antiserum or heat for 15 min and assayed.

Since isoagglutination with α-fla antiserum induces elevated levels of cAMP in vivo in wild-type and *imp-5* gametes (Table I), we asked whether cyclase activity was also stimulated. Wild-type and *imp-5* gametes were treated with α-fla antibody in vivo for 15 min, and flagellar and cell-body adenylyl cyclase preparations were then assayed in vitro. Flagellar cyclase activity and cell-body cyclase activity again both increase in response to adhesion (Table IV). To ask whether the cell-body cyclase can be directly stimulated by antiserum, flagella-less *bald 2 mt+* mutant gametes were first treated with GLE to remove their cell walls and then exposed to α-fla. In this case, the cell-body enzyme was not significantly stimulated (Table IV), indicating that the in vivo stimulation of the cell-body enzyme requires the presence of flagella and, presumably, flagellar adhesion.

Since the *imp-3* mutation affects cAMP production in vivo (Fig. 1 and Table I), it was expected that *imp-3* preparations would fail to display the adhesion-induced stimulation of flagellar cyclase activity. However, this was not the case. Fig. 2 *B* (●) shows that the *imp-3* flagellar enzyme activates during sexual adhesion, although not as robustly as the wild-type control, and Table IV documents that the enzyme activates as fully as wild type in response to α-fla presentation. On the other hand, the *imp-3* cell-body enzyme does not respond to either sexual or antibody adhesion (Fig. 2 *C*, ●, and Table IV). This suggests that the *imp-3* defect is interposed between the twofold activation of the flagellar enzyme and the activation of the cell-body enzyme, an inference supported by experiments presented in a later section.

Table V summarizes the effects of various upstream and downstream inhibitors of the mating reaction (Goodenough, 1993; Goodenough et al., 1993) on the adhesion-induced stimulation of cyclase activity. In these experiments we utilized the *imp-1 mt+* strain, which agglutinates and elevates cAMP levels normally (Pasquale and Goodenough, 1987) but fails to undergo cell fusion and disadhesion (Goodenough et al., 1982); use of the mutant prevents confusion between a drug-induced inhibition of cyclase activation and the natural inhibition of cyclase activation that accompanies

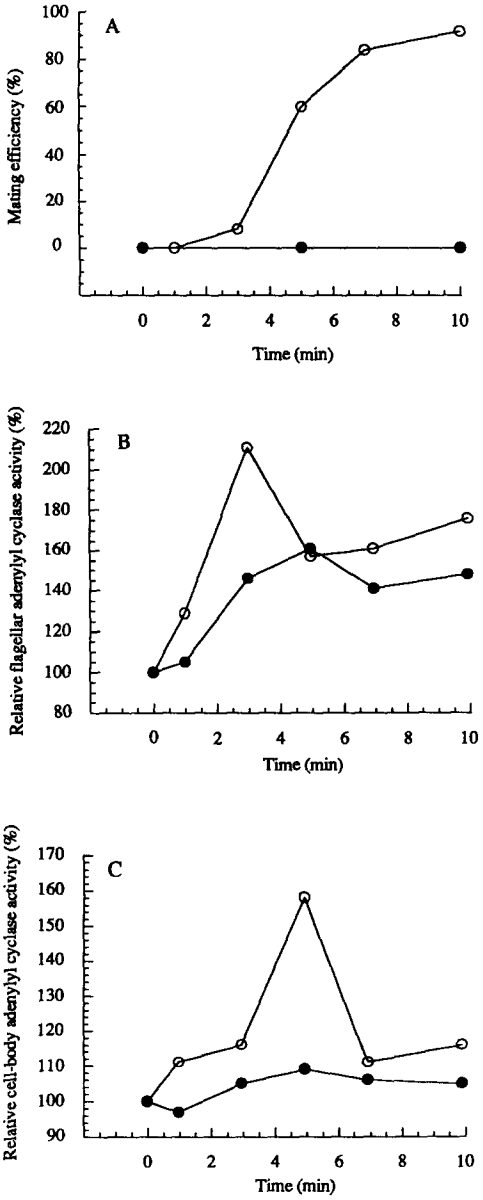

Figure 2. Time course of flagellar and cell-body adenylyl cyclase activation in wild-type and *imp-3* matings. Wild-type or *imp-3* gametes of both mating types were treated with GLE and mixed together. At the times indicated, 3-ml portions were mixed with dibucaine to obtain flagella, and 0.3-ml portions were freeze thawed to obtain cell bodies. The specific activity of the adenylyl cyclase from the unmated samples at 0 min is expressed as 100%. (*A*) Mating efficiency; (*B*) flagellar adenylyl cyclase activity; (*C*) cell-body adenylyl cyclase activity. Wild-type gametes (○); *imp-3* gametes (●).

disadhesion (see Figs. 1 and 2). None of the agents tested has a potent effect on adhesion-induced activation of the flagellar enzyme except Cd^{2+}, which completely blocks the stimulation at 100 μM. Similarly, 100 μM Cd^{2+} completely blocks the in vivo activation of the flagellar enzyme in wild-type matings (data not shown). In contrast, all of the upstream agents tested are inhibitory to the activation of the cell-body enzyme, whereas staurosporine, a downstream inhibitor, is not inhibitory. Thus upstream inhibitors such as lidocaine, TFP, and diltiazem appear to act between the twofold activation of the flagellar enzyme and the activation of the cell-body enzyme, the locus of the *imp-3* defect.

Table IV. In Vivo Activation of Adenylyl Cyclase by α-fla Antiserum

Strain	Percent increase of adenylyl cyclase activity	
	Flagella	Cell-body
Wild-type (*mt*$^+$)	40	34
imp-5 (*mt*$^+$)	71	48
bald-2 (*mt*$^+$)	N.A.	2
imp-3 mt$^+$	47	6

GLE-treated gametes (1 × 10^8 cells/ml) were mixed with α-fla antiserum (0.16 mg/ml) and incubated for 15 min. For flagellar adenylyl cyclase, flagella were isolated by dibucaine and freeze-thawed. For cell-body adenylyl cyclase, cells were freeze thawed. Values are averages from two different experiments.

Agglutinin-driven Cyclase Stimulation Is Not Seen In Vitro

To complement the analysis of flagellar cyclase activation in vivo, we sought to study adhesion-activation in vitro. The ideal system would be to harvest *mt*$^+$ and *mt*$^-$ gametic flagella separately, mix them together to induce their agglutinin-mediated adhesion (Goodenough, 1986), and then measure enzyme activation. For reasons that we fail to understand, however, no cyclase activation is observed. This negative result has been obtained with native flagella and with flagella from gametes pretreated with db-cAMP/IBMX to maximize their agglutinin levels (Goodenough, 1989). Moreover, it is obtained whether cyclase is assayed immediately after mixing or after more prolonged incubations. We have therefore developed two other approaches to monitor in vitro stimulation of the flagellar cyclase. These are described in the next two sections.

Cyclase Stimulation In Vitro by Soluble Cross-Linkers

As noted above, flagellar isoagglutination can be elicited by the polyclonal α-fla antiserum. It can also be elicited by the lectin Con A (McLean and Brown, 1974). Both reagents in-

Table V. Effect of Various Reagents on In Vivo Activation of Adenylyl Cyclase during Mating

Reagents	Percent increase of adenylyl cyclase activity	
	Flagella*	Cell body‡
No addition	205	44
Lidocaine	143	4
TFP	148	24
Diltiazem	129	23
Cd^{2+}	1	11
La^{3+}	178	14
Staurosporine	118	65

For all entries in table, each rate is the average of two experiments. Inhibitor concentrations are given in Materials and Methods.
* *Imp-1 mt*+ and *mt*− gametes were pretreated with inhibitors for 10 min. Half of each sample was allowed to mate for 5 min while the other continued to incubate without mating. Flagella were isolated by dibucaine.
‡ *Imp-1 mt*+ and *mt*− gametes were treated with GLE and then pretreated with inhibitors for 10 min. Half of each sample was allowed to mate for 5 min while the other continued to incubate without mating. Each sample was then freeze thawed and assayed.

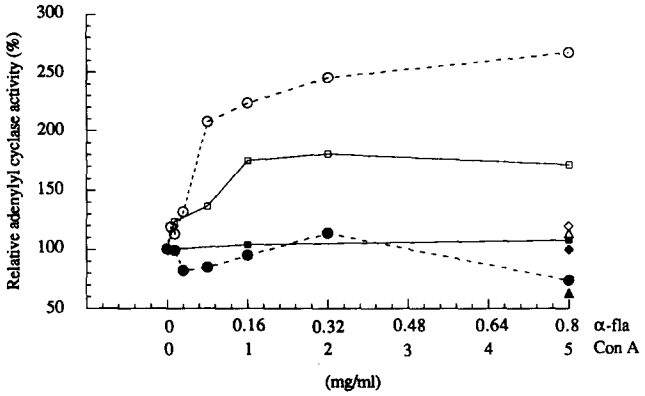

Figure 3. Effects of α-fla antiserum and Con A on flagellar and cell-body adenylyl cyclase activity. Freeze-thawed flagella and cell bodies were mixed with indicated concentrations of α-fla antiserum, pre-immune serum, or Con A, incubated for 15 min on ice, washed, resuspended in buffer A and assayed. Flagellar adenylyl cyclase activity: α-fla antiserum (□); pre-immune antiserum (◇); Con A (○); 0.1 M α-methylmannoside before Con A (△). Cell-body adenylyl cyclase activity: same as above only symbols are closed.

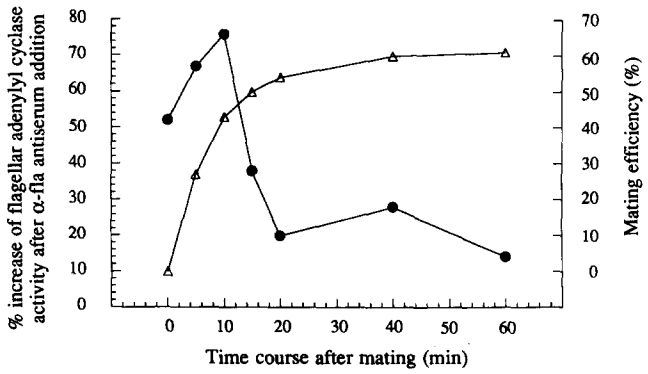

Figure 4. Loss of the flagellar adenylyl cyclase-activated system induced by α-fla antiserum during mating. Mt^+ and mt^- gametes (1×10^8 cells/ml) were mixed together at 0 min and flagella were isolated by pH shock at indicated times. To determine the activation capacity induced by the antiserum, each sample was incubated for 15 min on ice in the absence or presence of α-fla antiserum (0.17 mg/ml) and assayed as in Fig. 3. Percent increase of flagellar cyclase activity after antiserum addition (●); mating efficiency (△).

teract with numerous glycopolypeptide epitopes on flagellar surfaces (Adair et al., 1978; Monk et al., 1983), and both elicit mating responses only when functionally divalent (McLean and Brown, 1974; Goodenough and Jurivich, 1978; Mesland et al., 1980; Kooijman et al., 1989).

Fig. 3 shows the dose-response curve for activation of the gametic flagellar adenylyl cyclase by α-fla presentation in vitro. A 1.8-fold stimulation is effected, comparable to that observed when the antiserum is presented in vivo (Table IV), whereas no response is elicited in the pre-immune control. Fig. 3 also shows parallel data for Con A. The flagellar cyclase is stimulated 2.5-fold, and 100 mM α-methylmannoside completely blocks the stimulation.

That in situ cross-bridging is necessary for the twofold stimulation is indicated by the effects of lubrol solubilization (Table III, column 2). If flagellar membranes interact first with the antiserum and then with detergent, the activated state is preserved; in contrast, if the membranes are first solubilized in lubrol and then treated with α-fla, no stimulation takes place.

Table VI summarizes the in vitro cross-linking responsiveness of various *C. reinhardtii* strains. The first entries docu-

Table VI. Activation of Adenylyl Cyclase In Vitro by α-fla Antiserum, Con A and Heat

Flagellar source	Percent increase of flagellar adenylyl cyclase activity		
	α-fla	Con A	40°C
Wild-type gametes (mt^+)	76	32	1280
Wild-type gametes (mt^-)	58	66	2300
db-cAMP-treated gametes (mt^+)	54	39	ND
imp-1 gametes (mt^+)	54	45	ND
imp-5 gametes (mt^+)	58	ND	ND
imp-10 gametes (mt^-)	42	41	ND
imp-3 gametes (mt^+)	50	ND	183
imp-3 gametes (mt^-)	67	ND	312
Vegetative (mt^+)	59	0	−70
Vegetative (mt^-)	96	0	ND
Zygote (1 h after mate)	10	2	1772

2×10^8 flagella/ml were freeze thawed and incubated for 15 min on ice in the absence or the presence of 0.17 mg/ml α-fla antiserum or 1.0 mg/ml Con A or incubated at 40°C for 15 min. Each rate is the average of at least two experiments.

Table VII. Effect of Various Reagents on In Vitro Activation of Flagellar Adenylyl Cyclase Activity

Reagents	Percent increase of flagellar adenylyl cyclase activity		
	α-fla activation*	Before 40°C activation‡	After 40°C activation§
No addition	62	900	1,100
Dibucaine	78	20	−88
Lidocaine	66	340	380
TFP	58	−90	−88
Diltiazem	90	−70	260
Cd^{2+}	44	900	1,100
La^{3+}	38	290	308
EGTA	62	60	104
ATP	68	240	236
ATP+staurosporine	ND	120	212
ADP	ND	190	ND
AMPPNP	ND	270	140
GTP	ND	30	404
GDP	ND	120	ND
cAMP	ND	570	ND
AMP	ND	900	824
Staurosporine	42	700	1,052
Vanadate	60	560	772

For all entries in table, each rate is the average of at least two experiments. Concentrations of inhibitors are given in Materials and Methods; nucleotides were at 1 mM.

* Freeze-thawed mt^+ flagella (2×10^8 flagella/ml) were pretreated with inhibitors for 10 min and treated with α-fla antiserum (0.17 mg/ml) for 15 min on ice. After 13,000 *g* centrifugation, each sample was resuspended in buffer A containing inhibitors and assayed.

‡ Freeze-thawed mt^+ flagella (2×10^8 flagella/ml) were pretreated with inhibitors for 10 min on ice and then incubated at 40°C for 15 min. Each sample was washed in buffer A and assayed at 30°C.

§ Freeze-thawed flagella were incubated at 40°C for 15 min, cooled down, and then incubated for 15 min in the presence of inhibitors on ice. All samples were centrifuged, resuspended in buffer A and assayed at 30°C.

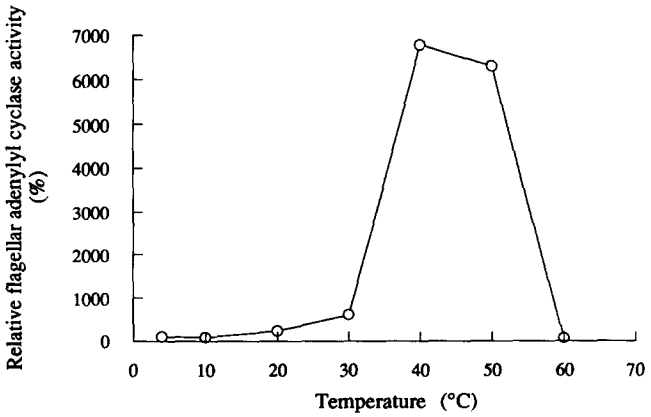

Figure 5. Effect of temperature on flagellar adenylyl cyclase. Freeze-thawed flagella were pretreated at the indicated temperatures for 15 min, harvested by centrifugation, resuspended in buffer A and assayed at 30°C for 10 min.

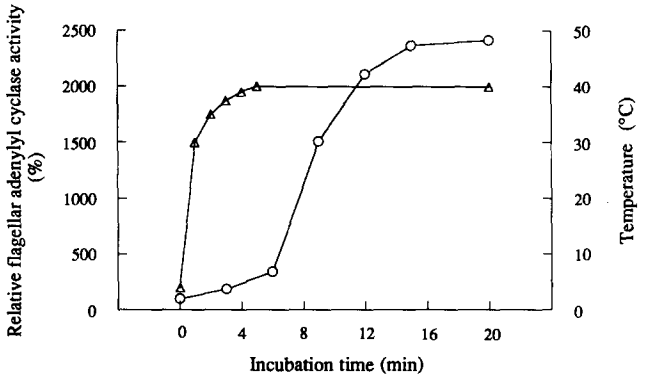

Figure 6. Time course of 40°C activation of flagellar adenylyl cyclase. Freeze-thawed flagella were pre-incubated at 40°C for the indicated times. Each sample was centrifuged, resuspended in buffer A, and assayed at 30°C. Flagellar adenylyl cyclase activity (○); temperature of sample (△).

ment that agglutinin levels per se are not important for the effect. Thus gametes treated with db-cAMP have eightfold more agglutinin than controls (Goodenough, 1989; Hunnicutt and Snell, 1990) whereas the *imp-5* and *imp-10* mutant strains are devoid of active agglutinins (Adair et al., 1983; Collin-Osdoby and Adair, 1985), yet all display comparable levels of flagellar cyclase stimulation by both α-fla and Con A. Consistent with its in vivo flagellar responsiveness to soluble crosslinkers (Table IV), moreover, the *imp-3* flagella also undergo an in vitro cyclase activation in response to these agents (Table VI).

In contrast, responsiveness to soluble cross-linkers shows two life-cycle-specific differences (Table VI). First, the flagellar cyclase of vegetative cells is activated by α-fla but not, interestingly, by Con A, indicating that Con A–binding glycosyl residues may associate with the relevant activating molecules at the time of gametic differentiation. Second, the cyclase of zygotic flagella is not stimulated by either α-fla or Con A. Fig. 4 shows the kinetics of this loss in zygotic inducibility. During the course of the mating reaction (first 20 min) the gametic enzyme actually increases its responsiveness to antibody, but concomitant with cell fusion, antibody stimulation is lost. We conclude, therefore, that the gametic flagellar cyclases lose their sensitivity to cross-linking at the time of zygote formation.

The gametic cell-body cyclase is not stimulated by either Con A or α-fla presentation in vitro (Fig. 3), even though the plasma membrane binds both ligands (Milliken and Weiss, 1984; Adair, 1985), consistent with its refractoriness to direct α-fla stimulation in vivo (Table IV).

Table VII, column 1 summarizes the effects of upstream and downstream inhibitors on in vitro α-fla–induced flagellar cyclase activation. None of the reagents tested shows a marked effect. In particular, Cd^{2+} is only somewhat inhibitory, in contrast to its dramatic inhibition of in vivo activation (Table V, column 1).

Cyclase Stimulation In Vitro by 40°C Incubation

Fig. 5 documents the second way that the gametic flagellar adenylyl cyclase can be stimulated in vitro: as first described by Zhang et al. (1991), the enzyme is strikingly activated (10–30-fold) if incubated at elevated temperatures and then assayed at lower temperatures. We have used throughout a 40°C incubation for 15 min followed by assay at 30°C.

The kinetics of activation are shown in Fig. 6. It takes 4 min for the flagellar suspensions to reach 40°C; activation begins at 6 min and reaches a maximum 20 min later. The extent of activation is variable: the 70-fold stimulation of Fig. 5 is particularly dramatic, the 10–20-fold of Fig. 6 and Table VI is more typical, and the 10-fold of Table VII is similar to the values obtained by Zhang et al. (1991).

Table III, column 3 documents that 40°C activation, in contrast to α-fla activation, is not stable to detergent. Thus the addition of 0.1% lubrol to flagella after 40°C exposure not only cancels the stimulatory effect but actually inhibits activity, in contrast to controls. Moreover, if the enzyme is first solubilized and then heated, it is also inactivated.

Table VI, column 3 displays 40°C activation data for various cell types and strains. Interestingly, zygotic cyclase can be 40°C stimulated to the same extent as gametic cyclase, in contrast to the zygotic loss of cyclase stimulation by cross-linking reagents (Table VI and Fig. 4). Reciprocally, the vegetative cyclase shows no 40°C stimulation, and in fact a 40°C inactivation, in contrast to its stimulation by cross-linking agents (Table VI). The cell-body cyclase is also insensitive to 40°C exposure (data not shown). And finally, Table VI documents that the *imp-3* flagellar adenylyl cyclase is defective in its ability to undergo 40°C activation: activation is ~25% of wild-type controls.

Table VII shows the effects of various reagents on 40°C activation when presented to the freeze thawed flagella before temperature elevation (column 2) or after temperature elevation (column 3). Shown first are several upstream inhibitors which block activation both before and after heating, in marked contrast to their failure to block activation by soluble cross-linkers and by in vivo adhesion (Table VII, column 1, and Table V). Diltiazem is more potent when added before than after heat treatment, whereas TFP and dibucaine are strongly inhibitory under both conditions. Strikingly, Cd^{2+} has no effect on 40°C activation, in contrast to its marked effect on twofold activation during mating (Table V).

The second sector of Table VII shows the effects of various nucleotides on 40°C activation. Zhang et al. (1991) report that the inclusion of ATP with an ATP-regenerating system virtually eliminates 45°C activation whereas the analog

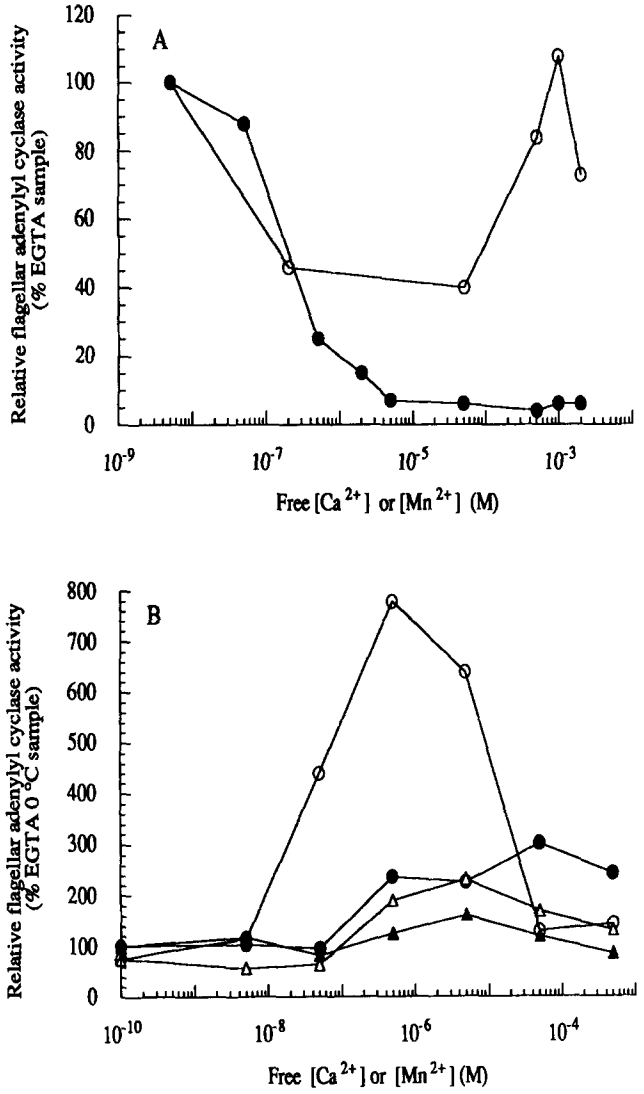

Figure 7. Effects of free calcium or manganese ions on flagellar adenylyl cyclase activity. (*A*) Exposure to cations during enzyme assay. Flagella (pH shock) were suspended in 1 mM EGTA, 10 mM Tris-HCl, pH 7.5, 5 mM $MgSO_4$, 1 mm DTT, and freeze thawed. $CaCl_2$ or Mn acetate were added at various concentrations and each sample was assayed for 10 min at 30°C. Ca^{2+} (●); Mn^{2+} (○). (*B*) Exposure to cations during 40°C incubation. Flagella (pH shock) were suspended in 1 mM EGTA, 10 mM Tris-HCl, pH 7.5, and 4% sucrose. $CaCl_2$ or Mn acetate were added at various concentrations and each sample was incubated on ice (0°) or at 40°C for 15 min. Each sample was then pelleted, resuspended in buffer A, and assayed for 10 min at 30°C. Ca^{2+}, 0°C (●); Ca^{2+}, 40°C (0); Mn^{2+}, 0°C (▲); Mn^{2+}, 40°C (△). For both *A* and *B*, free metal concentrations were calculated using the Max Chelator program (courtesy of Dr. Christopher Patton, Stanford University).

AMP-PNP has no inhibitory effect, results that they interpret to suggest that the cyclase is ordinarily inhibited by a heat-labile protein kinase and activated by a heat-stable protein phosphatase. Using different strains and a somewhat different buffer system, we confirm that ATP is inhibitory (~70%). However, in contrast to Zhang et al., the kinase inhibitor staurosporine does not prevent this ATP inhibition. Moreover, equivalent or greater levels of inhibition are also obtained using AMP-PNP, ADP, GTP, and GDP (but not

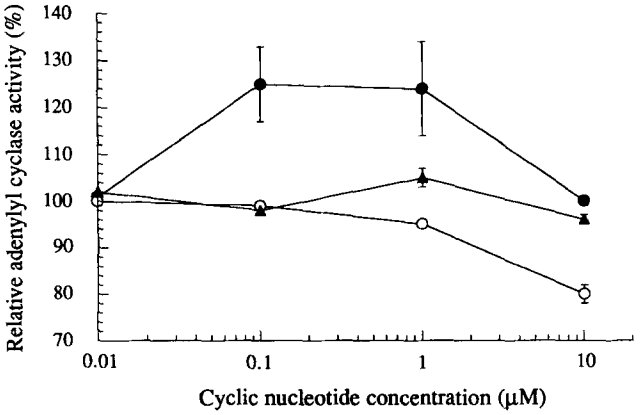

Figure 8. Effect of cyclic nucleotides on flagellar and cell-body adenylyl cyclase. The 400-*g* pellet from freeze-thawed cells was used for cell-body adenylyl cyclase. Flagella isolated by pH shock were freeze thawed. Each sample was assayed with indicated concentrations of cyclic nucleotides at 30°C for 10 min. Cell-body adenylyl cyclase activity in the presence of cAMP (●) or cGMP (▲); flagellar adenylyl cyclase activity in the presence of cAMP (○). Values are averages from three different experiments.

AMP, cAMP, or staurosporine alone). Like Zhang et al., we see no effect of vanadate on activation although vanadate is a potent inhibitor of flagellar protein phosphatase activity (Bloodgood, 1992). Taken together, our observations do not support the kinase/phosphatase model of cyclase activation, no do they rule it out. An alternative possibility, that the di- and trinucleotides are acting as chelating agents, is supported by the inhibitory effects of EGTA (Table VII) and EDTA (Zhang et al., 1991).

Ca^{2+} Effects on Flagellar Adenylyl Cyclase Activity

The inhibition of 40°C activation by EGTA and by TFP suggests the involvement of Ca^{2+}. Flagellar adenylyl cyclase activity per se is stimulated by EGTA: the basal rates in buffer A (no Ca^{2+} added) (Table II) are stimulated twofold by 1 mM EGTA. If this "EGTA rate" is set at 100, then the addition of free Ca^{2+} is strongly inhibitory (Fig. 7 *A*, ●). However, the experiments summarized in Fig. 7 *B* document that both basal (●) and heat-activated (○) adenylyl cyclase activity can be stimulated by prior exposure to Ca^{2+}. In these experiments, permeabilized flagellar samples were incubated for 15 min in increasing [Ca^{2+}] at either 0° or 40°C; they were then pelleted, resuspended in Ca^{2+}-free buffer A, and assayed at 30°C for adenylyl cyclase activity. Samples incubated at 0°C show a 2–3-fold stimulation after exposure to relatively high [Ca^{2+}] (>5 × 10^{-6} M). Strikingly, the 40°C samples undergo the 10-fold activation only in the [Ca^{2+}] range of 10^{-7}–10^{-6} M; heat-activation is disallowed at both lower and higher [Ca^{2+}]. That this effect is specific for Ca^{2+} is indicated by experiments with Mn^{2+}. The presence of millimolar Mn^{2+} stimulates basal flagellar cyclase activity (Pasquale and Goodenough, 1987, and Fig. 7 *A*), but it fails to substitute for Ca^{2+} in the heat-activation response (Fig. 7 *B*).

Stimulation of Cell Body Adenylyl Cyclase by cAMP

The stimulation of cell-body adenylyl cyclase in response to flagellar adhesion (Fig. 2 and Table IV) is intriguing because

the cell bodies make no direct adhesive contacts during the mating reaction. Therefore, activation of the cell-body enzyme must be the result of events stimulated by flagellar adhesion, an inference supported by the staggered kinetics of the two responses (Fig. 2), by the failure of antibody and Con A to directly stimulate the cell-body enzyme (Table IV and Fig. 3), and by the failure of the cell-body enzyme to activate in *imp-3* matings (Fig. 2) and in the presence of upstream inhibitors (Table V). An obvious possibility is that the cAMP generated by the adhesion-activated flagellar cyclase might diffuse into the cell body and stimulate the cell-body cyclase. Fig. 8 presents experiments which support this model: the cell-body enzyme is shown to be stimulated by cAMP, but not cGMP, in vitro, whereas the flagellar enzyme is either unaffected or inhibited by cAMP.

Discussion

How Many Kinds of Flagellar Cyclases?

The experiments presented in this paper document that flagellar adenylyl cyclase activity can be stimulated in two ways: the native adhesion reaction and soluble cross-linkers stimulate activity at least twofold, whereas 40°C exposure stimulates activity at least 10-fold. Each type of stimulation displays distinctive sensitivities to drugs, to Ca^{2+}, to gene mutation, and to life-cycle stages. Such data can be interpreted to indicate that a single flagellar enzyme is subjected to two levels of regulation, or that the flagellum possesses two species of cyclase, Type 1 and Type 2, each displaying distinctive sensitivities. Since Cd^{2+} reversibly blocks the overall mating reaction and the mating-induced elevation of cAMP levels in vivo (Goodenough et al., 1993) and selectively blocks the twofold activation, the activation of the Type 1 enzyme would, by this model, be necessary to activate the Type 2 enzyme. The issue cannot be settled until the relevant cyclase gene(s) have been cloned and their products localized. Since the well-characterized examples of cyclase regulation in mammalian systems entail a single enzyme modulated by multiple regulators (Tang and Gilman, 1992), we have adopted the single-enzyme model in the ensuing discussion, but the data are consistent with either possibility.

The Twofold Cyclase Stimulation

The flagellar cyclase is stimulated twofold when gametic flagella are harvested during the in vivo mating reaction and assayed in vitro (Fig. 2), or when gametes of one mt are isoagglutinated in vivo with the α-fla antiserum and then assayed in vitro (Table IV). It is also stimulated twofold when isolated flagella of a single mating type are incubated in vitro with either Con A or α-fla (Fig. 3). We propose that the twofold activation of cyclase by adhesion in vivo and by cross-linkers in vitro reflect the same underlying change in the properties of the enzyme: both are insensitive to dibucaine, TFP, EGTA, and lubrol; both are unaffected by the *imp-3* mutation; and both are displayed by the vegetative enzyme but lost at the time of zygotic cell fusion.

The obvious model to explain these observations is that the extracellular cross-bridging of flagellar cyclases somehow stimulates their intracellular catalytic domains, the precedent being the activation of receptor kinases by external cross-linking events (Ullrich and Schlessinger, 1990). This model cannot be elaborated further, however, until more is known about the molecular structure of the flagellar cyclases.

The twofold activation is selectively blocked by Cd^{2+} (Table V), a potent inhibitor of Ca^{2+} channels in vertebrate systems (Lansman et al., 1986), and the basal enzyme can be activated twofold by exposure to relatively high concentrations of free Ca^{2+} (Fig. 7 *B*). Bloodgood and associates have presented evidence that the cross-linking of flagellar surface ligands in vegetative cells generates an increased $[Ca^{2+}]$ in the flagellar matrix (Bloodgood and Salamonsky, 1990, 1991; Bloodgood, 1992). Taken together, these observations suggest a role for Ca^{2+} in the twofold activation process, but this role must be indirect since the presence of free Ca^{2+} is itself inhibitory to cyclase activity (Fig. 7 *A*).

Loss of Twofold Cyclase Stimulation with Zygote Formation

At the time of zygote formation, three functions are lost: the flagella lose their native agglutinability, they lose the mating-induced twofold stimulation of the cyclase (Fig. 2), and they lose the crosslinker-induced twofold stimulation of the cyclase (Table VI and Fig. 4). Importantly, Hunnicutt and Snell (1991) have shown that zygotic flagella retain abundant agglutinin proteins which are nonadhesive in situ but fully adhesive if they are extracted and dried down on a glass surface. Possibly, therefore, agglutinins are adhesive only if they are able to associate locally ("patch") in the membrane (Demets et al., 1988; Tomson et al., 1990; Hunnicutt and Snell 1991) or if they are similarly concentrated on a glass surface (Adair et al., 1982), and zygotic cell fusion may eliminate this capability, manifested as well by the inability of the cyclase to associate locally and thereby activate.

The gametic cell-body adenylyl cyclase is membrane-associated, presumably with the plasma membrane, yet it is not direct stimulated by lectins or antibody (Table IV and Fig. 3). Interestingly, the gametic plasma membrane also carries abundant agglutinin proteins which are nonadhesive in situ but fully adhesive if they are extracted and dried down on a glass surface (Hunnicutt and Snell, 1991). Therefore, the plasma membrane may share with the zygote flagellar membrane an inability both to patch agglutinins and to activate cyclases by cross-bridging.

The 10-fold Cyclase Stimulation

We have confirmed the intriguing observation of Zhang et al. (1991) that the gametic flagellar cyclase can be stimulated up to 70-fold, and routinely 10-fold, by 40°C incubation in vitro (Fig. 5). Zhang et al. (1991) explain the activation phenomenon by proposing a protein kinase/phosphatase "switch." As detailed in Results, however, we have been unable to reproduce some of the observations that generated this model, and we therefore propose an alternative. In the alternate model, adhesion in vivo causes a protein (C) to adopt an activated state (C^*) which in turn catalyzes a 10-fold activation of the flagellar adenylyl cyclase. The cyclase may also undergo an adhesion-stimulated change ($Cy \rightarrow Cy^*$) such that it is susceptible to C^* activation. The in vivo-generated C^* and/or Cy^* states are proposed to be labile such that, when agglutinated flagella are subsequently assayed in vitro, only the stable, twofold, C-independent cyclase stimulation is detected. In contrast, in vitro exposure to 40°C is proposed to

generate a stable C* and/or Cy* configuration, allowing detection of the 10-fold, C-dependent cyclase stimulation.

Three sets of observations can be considered in the context of this alternate model. (*a*) Certain upstream inhibitors which block the adhesion-driven cAMP elevation in vivo (Goodenough et al., 1993) block the 10-fold activation (Table VII). Of these, TFP and dibucaine can block the stimulated rates when added after 40°C exposure, indicating that they may affect the postulated ability of C* to stimulate the cyclase, whereas other inhibitors (e.g., diltiazem) are more effective when added before 40°C exposure, indicating that they may block the postulated C→C* and/or Cy→Cy* transition. Importantly, EGTA is equally inhibitory in both cases, and exposure to micromolar Ca^{2+} is necessary for heat activation (Fig. 7 *B*), suggesting that C might be a calcium-sensitive protein such as calmodulin or centrin (see also Goodenough, 1993). (*b*) The vegetative cyclase does not display 40°C activation; in fact, it is heat inactivated (Table VI). This suggests that the vegetative cyclase is a different gene product from the gametic cyclase and/or that the postulated C-system becomes coupled to the cyclase during gametic differentiation to catalyze the large cAMP burst needed to signal mating responses. (*c*) The *imp-3* mutation, which blocks signal transduction in vivo, inhibits the 40°C activation by 80% (Table VI), even though basal and twofold cyclase rates are normal (Fig. 2 and Tables II and VI). The *imp-3* mutation may affect the postulated C-sytem itself; alternatively, it may alter the flagellar cyclase such that it cannot adopt the Cy* configuration and hence cannot be stimulated properly by the postulated C*. In either case, the *imp-3* mutation is the first to be reported that specifically blocks gametic signal transduction. The temperature-sensitive *gam-1* mutant, while also defective in signaling, has a pleiotropic phenotype which also includes a defective cell-fusion apparatus (Forest and Togasaki, 1975; Forest et al., 1978; Forest and Ojakian, 1989; Goodenough, 1989).

Sequential Activation

Our working model of the cAMP response in *C. reinhardtii* is that it proceeds in three steps. (Step 1) A twofold stimulation of flagellar cyclase activity, stimulated by the adhesive "patching" of agglutinins or other surface ligands, is selectively blocked by Cd^{2+} and stable to in vitro assay. (Step 2) A 10-fold stimulation of flagellar cyclase activity, stimulated by a Ca^{2+}-dependent C→C* activation, is labile to in vitro assay but mimicked by 40°C exposure *in vitro*; this is gamete-specific, blocked by the *imp-3* mutation, and vulnerable to such inhibitors as TFP, EGTA, and dibucaine. (Step 3) An approximately twofold stimulation of the cell-body adenylyl cyclase follows full activation of the flagellar cyclase (Fig. 2 *C*).

The following observations suggest that the three steps represent an interdependent cascade. (*a*) Cd^{2+} completely inhibits all mating responses (Goodenough, 1993; Goodenough et al., 1993) even though it only blocks step 1, suggesting that step 1 initiates the cascade. (*b*) Several inhibitors, such as TFP, that block mating responses and full-scale cAMP elevation in vivo (Pasquale and Goodenough, 1987), are without effect on step 1 but block 40°C activation, the proposed in vitro manifestation of Step 2. Similarly, the *imp-3* mutation is without effect on step 1 but blocks step 2 and all mating responses. Finally, the capacity to undergo step 2 is displayed by the gametic but not the vegetative flagellar enzyme. Taken together, these observations indicate that step 2 is necessary to the cascade, and while we have no direct evidence on this point, we postulate that step 1 is required to initiate step 2. (*c*) The dependence of step 3 (the activation of the cell-body cyclase) on steps 1 and 2 is supported by the fact that step 3 is blocked by all pharmacological inhibitors of flagellar cyclase activation and by the *imp-3* mutation (Fig. 2 *c* and Table V). Since the cell-body cyclase is stimulated in vitro by cAMP itself (Fig. 8), one possibility is that when the flagellar enzyme is stimulated 10-fold (step 2), it generates sufficient cAMP to diffuse into the cytosol and exceed the threshold for cell-body activation. Because of its large capacity, activation of the cell-body enzyme would produce the bulk of the cAMP necessary to complete the mating reaction.

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Note Added in Proof. While this manuscript was under review, Zhang and Snell (1993) independently reported that the vegetative flagellar cyclase is inhibited by heat and that gametic cyclase activity is inhibited by Ca^{2+}.

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