Cyclic AMP Functions as a Primary Sexual Signal in Gametes of *Chlamydomonas reinhardtii*

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Abstract. When *Chlamydomonas reinhardtii* gametes of opposite mating type are mixed together, they adhere by a flagella-mediated agglutination that triggers three rapid mating responses: flagellar tip activation, cell wall loss, and mating structure activation accompanied by actin polymerization. Here we show that a transient 10-fold elevation of intracellular cAMP levels is also triggered by sexual agglutination. We further show that gametes of a single mating type can be induced to undergo all three mating responses when presented with exogenous dibutyryl-cAMP (db-cAMP). These events are also induced by cyclic nucleotide phosphodiesterase inhibitors, which elevate endogenous cAMP levels and act synergistically with db-cAMP. Non-agglutinating mutants of opposite mating type will fuse efficiently in the presence of db-cAMP. No activation of mating events is induced by calcium plus ionophores, 8-bromo-cGMP, dibutyryl-cGMP, nigericin at alkaline pH, phorbol esters, or forskolin. H-8, an inhibitor of cyclic nucleotide-dependent protein kinase, inhibits mating events in agglutinating cells and antagonizes the effects of cAMP on non-agglutinating cells. Adenylate cyclase activity was detected in both the gamete cell body and flagella, with the highest specific activity displayed in flagellar membrane fractions. The flagellar membrane adenylate cyclase is preferentially stimulated by Mn^{++}, unresponsive to NaF, GTP, GTPγS, AlF_4^-, and forskolin, and is inhibited by trifluoperazine. Cyclic nucleotide phosphodiesterase activity is also present in flagella. Our observations indicate that cAMP is a sufficient initial signal for all of the known mating reaction events in *C. reinhardtii*, and suggest that the flagellar cyclase and/or phosphodiesterase may be important loci of control for the agglutination-stimulated production of this signal.

TRANSMEMBRANE signaling events that are triggered by direct cell–cell contact or by contact of cells with extracellular matrix materials are important regulatory phenomena. Cellular activities such as differentiation, growth, motility, and fusion are responsive to contact-mediated stimuli (reviewed in references 26 and 44). Most of our knowledge of transmembrane signaling has come from the study of cellular responses to soluble ligands such as hormones (3) and neurotransmitters (63), and much is understood about the coupling and regulation of ligand-occupied receptors to intracellular effectors such as adenylate cyclase (29) and phospholipase C (18). The extent to which these mechanisms apply to cell contact–mediated and extracellular matrix–mediated transmembrane signaling is largely unknown. Studies of immune cell (13), platelet (19), *Dictyostelium* (50), and sperm–egg interactions (76) have begun to address these questions.

The mating reaction of the unicellular, biflagellate alga *Chlamydomonas reinhardtii* is a phenomenon well suited to the study of contact-mediated signaling. Mating consists of a coordinated series of morphologically definable events that include membrane surface motility, secretion, and actin and tubulin polymerization. Gametes of opposite mating type (mt^+ and mt^-) first interact via sex type–specific glycoproteins termed agglutinins, which are associated with the flagellar surface. The agglutinin molecules have properties common to extracellular matrix components: they are extended rods bearing globular domains and are rich in hydroxyproline (1, 20, 31). After agglutination, the sequence of events listed below rapidly ensues: (*a*) Tipping: migration and localization of the initially random flagellar contact sites to the flagellar tip (33, 41). (*b*) Flagellar tip activation (FTA):[1] an accumulation of fibrous material in the interior of the flagellar tip accompanied by elongation of the doublet "A" microtubules of the axoneme (53). (*c*) Secretion: release of a cell wall autolytic enzyme ("autolysin") which effects shedding of the glycoprotein-rich cell wall (15, 35, 67). (*d*) Mating structure activation (MSA): a morphological transformation of the gametic mating structure which, in plus gametes, is accompanied by actin polymerization and results in a 1–4-μm-long apical extension called the fertilization tubule (24, 35); in minus gametes a smaller dome-shaped swelling is formed (32). (*e*) Cell fusion: Activated mating structures fuse (78),

1. *Abbreviations used in this paper*: CaM, calmodulin; db-cAMP, dibutyryl-cAMP; DIC, differential interference contrast; FTA, flagellar tip activation; H-8, N-[2-(methylamino)ethyl)]-5-isoquinolinesulfonamide; IBMX, isobutylmethylxanthine; MSA, mating structure activation; TFP, trifluoperazine.

followed by cell body confluence and flagellar disadhesion. The result is a quadriflagellated cell which ultimately resorbs its flagella and differentiates as a zygote (54).

The intracellular messenger(s) that elicit these mating events in response to flagellar adhesion have to date been unidentified. Several studies have presented evidence suggesting the involvement of calcium as a sexual signal in *C. reinhardtii* (10, 46, 68). More recently, in the related species *C. eugametos*, Pijst et al. (59) reported a mating-associated increase in intracellular cAMP content, but its relevance to sexual signaling was not determined.

The purpose of the present study was to investigate the potential connection of cAMP to sexual signaling in *C. reinhardtii*. We first established that mating-associated changes in intracellular cAMP also take place in this species. We next demonstrated that cAMP can initiate the mating events of tipping, flagellar tip activation, cell wall loss, mating structure activation, and cell fusion. Finally, we determined that gametic flagella possess both adenylate cyclase and cAMP phosphodiesterase activity, consistent with the possibility that one or both enzymes is directly affected by sexual adhesion.

Materials and Methods

Strains and Culture Conditions

Clones of the wild-type strain 137c, mt^+ and mt^-, of *C. reinhardtii*, which exhibit high mating efficiencies, were used in most experiments. The mutant strains *imp-1* mt^+ (32, 35), *imp-5* mt^+ (7), *imp-2* mt^+ (7), *imp-12* mt^- (42), *pf-18* mt^- (77), and *bald-2* mt^+ (34) were used as indicated. All strains are available from the *Chlamydomonas* Genetics Center, Duke University, Durham, North Carolina. Plate gametes (51) were harvested after 7–14 d on Tris-acetate-phosphate (TAP)-agar plates and suspended in nitrogen-free high-salt minimal medium (51) for 1–2 h until sexual signaling capacity had developed as evidenced by widespread wall loss within 30 s of mixing mt^+ and mt^- gametes. Mating efficiency (% cell fusion) was determined by counting biflagellate cells (BFC) and quadriflagellate cells (QFC) in fixed samples after a 20 min mating of equal numbers of mt^+ and mt^- gametes and applying the formula: Percent cell fusion = (2 QFC × 100)/2 QFC + BFC. Mating efficiencies were generally >90%.

Cyclic Nucleotide Assay

cAMP and cGMP were analyzed by radioimmunoassay based on the method of Steiner et al. (68). For the analysis of cAMP, ^{125}I succinylated cAMP tyrosine methyl ester was used as competing ligand, and highly specific commercial antiserum (New England Nuclear, Boston, MA) was employed. 0.5-ml samples of mating cells at 2×10^7 cells/ml were pelleted by a 3-s microfuge spin. Supernatants were rapidly aspirated and the cell pellet dispersed in 1.0 ml of 6% TCA. To each sample, 0.1 pmol of [^{3}H]cAMP was added as a recovery marker. Samples were frozen and thawed three times and insoluble debris was pelleted by microfuge spin. The supernatants were extracted three times with water-saturated ether and the aqueous phase was lyophilized. The lyophilate was dissolved in 1 ml of 50 mM sodium acetate buffer (pH 6.2), and 0.1-ml aliquots were taken for liquid scintillation and for radioimmunoassay. Samples and standards were acetylated using acetic anhydride and triethylamine to achieve an assay sensitivity of 0.01 pmol cAMP per assay tube. In preliminary experiments, the specificity of the assay was established by determining that the reactive compound in samples was susceptible to phosphodiesterase treatment (12).

Assay of cGMP was as described above, with the substitution of succinyl cGMP tyrosine methyl ester-[^{125}I] as competing ligand, and specific commercial antiserum (New England Nuclear). Cross reactivity between cAMP and cGMP in the radioimmunoassay is <0.004%.

Cell Fractionation

Gametes harvested from 180 plates were washed twice in 10 mM Tris (pH 7.4), pelleted at 3,000 *g*, and resuspended in cold 10 mM Tris-7% sucrose (pH 7.4). Deflagellation was carried out at 4°C by the pH-shock method of Witman et al. (80). Cell bodies were pelleted by brief spin at 2,000 *g*. The supernatant, which contained intact flagella, was layered over a cushion of 25% sucrose and spun at 3,000 *g* for 15 min to remove residual cell bodies. Flagella were aspirated from the sucrose cushion and pelleted at 17,000 *g* for 10 min, followed by resuspension in 30 mM Hepes, 5 mM $MgSO_4$, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 25 mM KCl, pH 7.4. For adenylate cyclase assays, whole cells, cell bodies, and detached flagella were sonicated (Microprobe Sonic Demembranator; Fisher Scientific Co., Pittsburg, PA) by three 15-s bursts at 0.5 relative output. For preparation of flagellar membrane vesicles, the detached flagellar suspension was frozen and thawed twice and then sheared through a 23-gauge hypodermic needle. After a 17,000 *g* spin to pellet naked axonemes, the vesicle-containing supernatant was spun at 100,000 *g* for 60 min to pellet the vesicles. The vesicle fraction was monitored by SDS-PAGE analysis and quick-freeze, deep-etch electron microscopy as described by Heuser (37).

Adenylate Cyclase Assay

Adenylate cyclase activity was determined by measuring the conversion of α[^{32}P]ATP to [^{32}P]cAMP, essentially according to the method of Salomon (61). The reaction mixture (0.1 ml) contained 25 mM Tris acetate buffer (pH 7.6), 0.5 mM ATP, 0.05 mM cAMP, 1 mM DTT, 0.1 mg/ml BSA, either 5 mM Mg acetate or 0.5 mM $MnCl_2$, and an ATP-regenerating system consisting of 5 mM creatine phosphate and 50 U/ml creatine phosphokinase. α[^{32}P]ATP (sp act 30 Ci/mmol; New England Nuclear) was present at 1×10^6 cpm per sample. For routine assays, sample protein was added at a final concentration of 0.1 mg/ml, and 10 mM theophylline was present. GTP (0.01 mM) was included for some experiments. Reactions were initiated in triplicate by adding sample protein to the prewarmed reaction mixture and allowed to proceed for 10 min at 37°C. The reaction was stopped by addition of 0.2 ml of a mixture of 2% SDS, 45 mM ATP, and 13 mM cAMP. After stopping, 10,000 cpm of [^{3}H]cAMP (sp act 12 Ci/mmol; New England Nuclear) was added to each sample as an internal recovery standard. Samples were heated in a boiling water bath for 4 min and 1 ml of distilled water was added to each.

[^{32}P]cAMP was isolated by decanting the sample tube into a column (0.5 × 15 cm) containing 2 ml of Dowex AG resin (50W-X4, 200–400 mesh; Sigma Chemical Co., St. Louis, MO). The eluate from this and two washes with 1 ml of distilled water was discarded. 3 ml of distilled water was then added to the column and the eluate passed directly through a second column containing 0.5 g of neutral alumina (type WN-3; Sigma Chemical Co.). [^{32}P]cAMP was eluted from the alumina with 5 ml of 0.1 M imidazole buffer (pH 7.3) directly into scintillation vials containing 15 ml of ACS scintillant (Amersham Corp., Arlington Heights, IL). Samples were counted on a Tri-Carb scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Recovery of [^{32}P]cAMP averaged 85% and blank values were <0.001% of the ^{32}P counts added to each tube. Protein concentrations were determined by the method of Bradford (11), using BSA as a standard.

Enzyme activity is reported as picomoles cAMP per minute per milligram protein. Reactions were linear with respect to both time (up to 20 min) and protein concentration (up to 40 μg per assay). Adenylate cyclase activity of cell fractions was stable at −70° for at least 30 d. Sonication of flagellar membrane vesicles was found to have no effect on their adenylate cyclase activity.

Cyclic Nucleotide Phosphodiesterase Assay

Cyclic nucleotide phosphodiesterase was assayed by monitoring the consumption of substrate cAMP using a modification of the method of Thompson et al. (73). Assay mixtures (0.2 ml) consisted of 40 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, and 50 pmol of cAMP. This solution was found to be compatible with the in vitro agglutination of isolated flagella (30), as monitored by phase-contrast microscopy. Reactions were initiated by adding intact flagella (5 or 50 μg protein) to assay mix prewarmed to 30°C. Triplicate reactions were run for 10 min and stopped by addition of 0.5 ml of 6% TCA. Samples were processed for radioimmunoassay of remaining cAMP as described above. Cross-reactivity between 5′-AMP and cAMP was <0.02%.

Microscopy

Light Microscopy. Assays for cell wall loss and fertilization tubule activation were conducted by direct visual counts of glutaraldehyde (1%) fixed cells in suspension. A Zeiss Axiomat microscope equipped with differential interference contrast optics was used. For each sample, 200–300 randomly

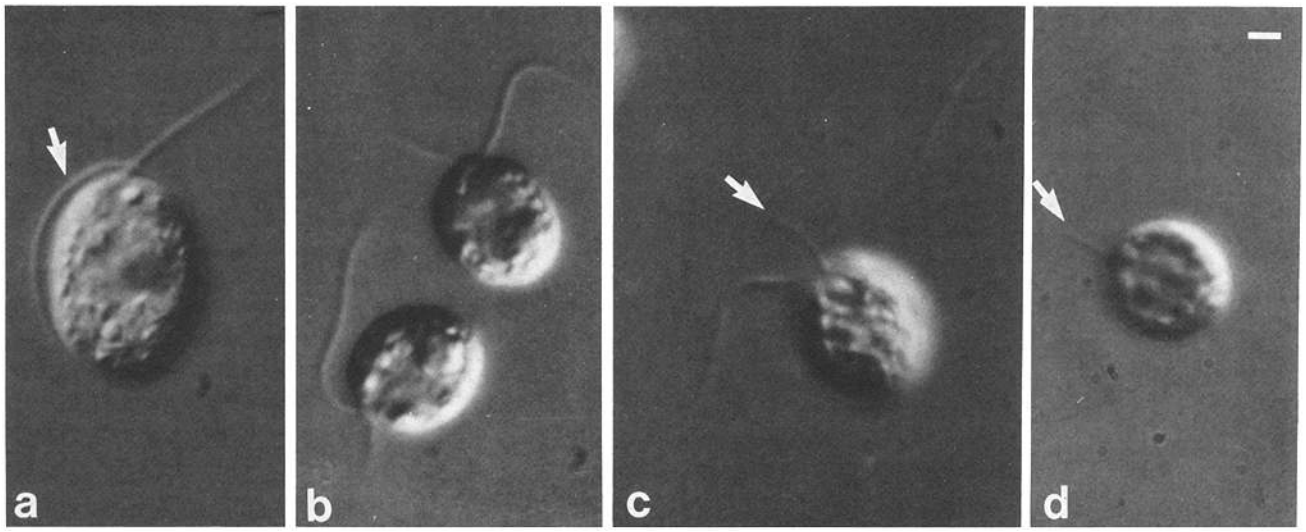

Figure 1. Gametes scored for mating phenotypes by DIC microscopy. (*a*) Gamete encircled by a cell wall (*arrow*). (*b*) Two gametes lacking cell walls. (*c*) Wild-type mt^+ gamete bearing a fertilization tubule (*arrow*). (*d*) Flagella-less *bald-2* mt^+ gamete, activated by db-cAMP + IBMX, bearing a fertilization tubule (*arrow*). Bar, 1 μm.

encountered cells were scored at a magnification of 1,250. Fig. 1, *a* and *b* shows gametes with and without walls: the distinct refractile wall (*arrow*) encircling the cell body is readily scored. Fig. 1 *c* shows a plus gamete with an activated mating structure (*arrow*); the 1–4-μm-long fertilization tubule located between the flagella is also readily scored. Neither the cell wall nor the fertilization tubule can be visualized by phase-contrast optics. Tipping was monitored by observation of the movement and localization of polystyrene microspheres (0.35-μm diam) on the flagella of the paralyzed flagellar mutant *pf-18* using standard phase-contrast optics (8).

Electron Microscopy. Cells were fixed and processed for thin-section electron microscopy as described (32).

Reagents

db-cAMP, 8-bromo-cGMP, GTPγS, phorbol myristate acetate, isobutylmethylxanthine (IBMX), papaverine, theophylline, phosphodiesterase, creatine, and creatine phosphokinase were purchased from Sigma Chemical Co. Ionomycin, A23187, nigericin, and forskolin were obtained from Calbiochem-Behring Corp., La Jolla, CA. *N*-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) was obtained from Seikagaku, Inc., St. Petersburg, FL.

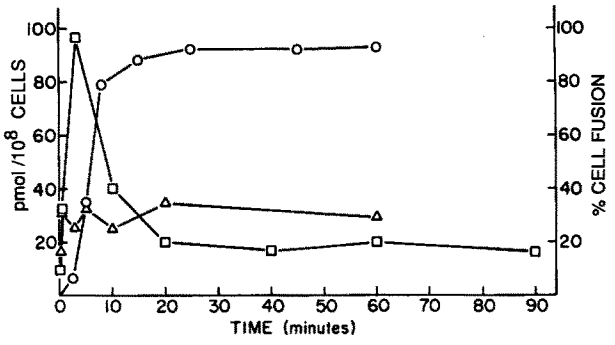

Figure 2. Kinetics of the cAMP (□) and cGMP (△) responses during mating. Wild-type mt^+ gametes at 2×10^7/ml in nitrogen-free high-salt minimal medium were added to an equal number of wild-type mt^- gametes. At the indicated times, aliquots of mating cells were either fixed with an equal volume of 2% glutaraldehyde (for determination of % cell fusion, ○), or pelleted and quenched with an equal volume of 6% TCA. Samples were processed for cyclic nucleotide determination as described in Materials and Methods. Data points represent the average of triplicate assays from two separate experiments.

Results

Cyclic Nucleotide Responses during Mating

As a first step in investigating whether cyclic nucleotides might be functioning as a sexual signal in *C. reinhardtii*, we examined cAMP and cGMP levels in mating gametes. Fig. 2 shows the time course of changes in cAMP and cGMP levels during mating in populations of wild-type gametes. Wild-type cells experienced a transient 10-fold increase in cAMP content peaking at or before 3 min of mating. The return of cAMP content to basal levels by 20 min coincides with completion of mating by the slowest members of the gamete population (Fig. 2). (Mating in gamete populations is not completely synchronous; some individual pairs are able to complete cell fusion within 1 min, whereas other pairs may require as long as 15 min.) cGMP levels displayed a less dramatic pattern of change during mating, increasing no more

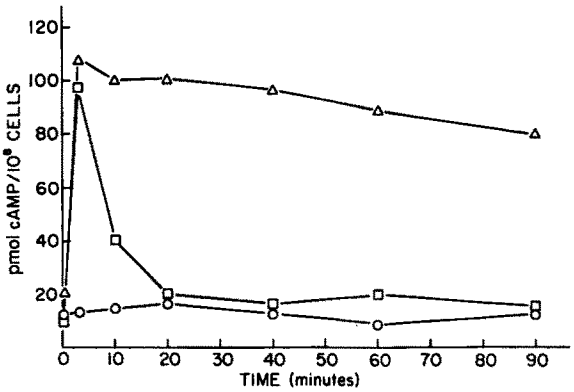

Figure 3. Kinetics of the cAMP response in mating-defective mutant gametes. Wild-type mt^- gametes at 2×10^7/ml were mixed with an equal number of either wild-type mt^+ (□), *imp-1* mt^+ (△) or *imp-5* mt^+ (○). At the indicated times, cells were pelleted, quenched with an equal volume of 6% TCA, and processed for cAMP determination. Data points represent the average of triplicate assays from two separate experiments.

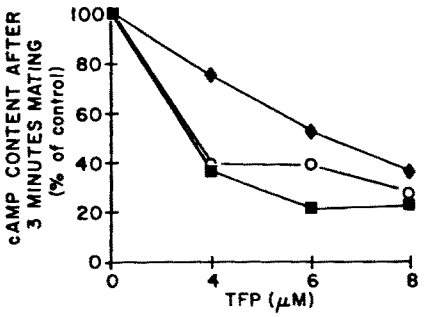

Figure 4. Effect of TFP on cAMP content during mating. Gametes at 0.5 × 10^7 cells/ml (□), 1 × 10^7 cells/ml (○), or 2 × 10^7 cells/ml (◇) were mated for 3 min in the presence of the indicated TFP concentrations. Cells were then pelleted, quenched with an equal volume of 6% TCA, and processed for cAMP analysis.

than twofold and remaining elevated throughout the mating period (Fig. 2).

Markedly different cAMP responses were observed with mating-defective mt^+ mutants. The *imp-5* mt^+ mutant, which is unable to agglutinate as a consequence of defective flagellar agglutinin synthesis (2), triggered no response either in itself or in its wild-type minus partners (Fig. 3). The *imp-1* mt^+ mutant, which can agglutinate but is unable to fuse as a consequence of a defective mating structure (32), undergoes a prolonged agglutinative interaction with its wild-type minus partners, accompanied by a prolonged elevation of cAMP (Fig. 3). Thus the rise in cAMP requires sexual adhesion, normally falls with flagellar disadhesion, and is prolonged when the flagella fail to disadhere.

Detmers and Condeelis (23) have recently reported that trifluoperazine (TFP), a calmodulin antagonist, blocks all post-adhesion mating events in a dose-dependent manner. Fig. 4 shows that similar concentrations of TFP also block the mating-associated rise in cAMP levels in a dose-related fashion.

Response of Non-Mated Gametes to Exogenous cAMP

We next asked whether gametes would display mating-specific responses, in the absence of adhesion, when presented with agents that elevate intracellular levels of cAMP. As illustrated by differential interference contrast (DIC) microscopy in Fig. 1, *b* and *c*, and quantitated in Table I, dibutyryl cAMP (db-cAMP) causes plus gametes to shed their walls and activate their mating structures in a dose-related fashion. Significant response to 1 mM db-cAMP required up to 3 h of incubation, an interval that could be shortened to 25 min by using relatively high concentrations (10–50 mM) of external db-cAMP. Such concentrations, while somewhat higher than the range (1–2 mM) normally used for in vivo studies (65), presumably reflect, at least in part, the relative impermeability of *C. reinhardtii* to many exogenous agents. Neither 5′-AMP nor adenosine, at similar concentrations, elicited any wall loss or mating structure activation.

The response to exogenous db-cAMP was augmented by the inclusion of cyclic nucleotide phosphodiesterase inhibitors (4). As shown in Table I, 1 mM and 10 mM db-cAMP produced much greater responses during the 25-min incubation in the presence of the methylxanthine IBMX. Pursuing this further, it was found that IBMX alone induced a dose-dependent activation of sexual signals (Table I), and also caused sevenfold elevation of endogenous cAMP (Fig. 5). Similar results were obtained with a second methylxanthine derivative, theophylline, at 5 mM (data not shown). That these effects were not unique to methylxanthines was shown by the fact that papaverine, an isoquinoline alkaloid that also inhibits cAMP phosphodiesterase (14), also elicited mating responses by itself and produced synergism with db-cAMP (Table I). The activation of wall loss and mating structures induced by db-cAMP plus IBMX was antagonized in a dose-dependent and reversible fashion by H-8, an inhibitor of cyclic nucleotide-dependent protein kinase (38) (Table I).

The earliest visible consequence of sexual adhesion in *C. reinhardtii* is the migration of agglutinated foci to the tips of the gametic flagella. To ask whether such tipward migration of surface molecules could be induced by IBMX + db-cAMP in the absence of adhesion, *pf-18* gametes pretreated with db-cAMP + IBMX were presented with polystyrene microspheres to assess their patterns of surface motility (8). The microspheres bound to the flagellar surfaces and moved baseward and tipward in an apparently normal fashion. Any microspheres that advanced to the tip itself, however, remained immobilized, so that after 15 min, most of the gametes carried a microsphere at one or both flagellar tips. Specifically, within 5 min after bead presentation, 8 out of 25 treated gametes carrying a bound microsphere carried the microsphere at a flagellar tip; within 10 min, 46 out of 65 were at a tip; and after 15 min, 60 out of 70 were at a tip. In contrast, only 1 out of 75 control gametes carried a tipped microsphere after 15 min. This same tipping pattern of microsphere motility occurs during the native mating reaction (41).

Additional features of the gametic response to IBMX and IBMX + db-cAMP were documented by electron microscopy. Fig. 6 shows that the induced plus fertilization tubules are filled with actin filaments, as in a natural mating (24, 35), and Fig. 7 documents that minus gametes are also stimulated to activate their mating structures, again in a fashion indistinguishable from normal mating (32). Fig. 8 shows that IBMX and IBMX + db-cAMP can also elicit FTA, with the response morphologically identical to the FTA induced during mating (53): a dense fibrous material accumulates between

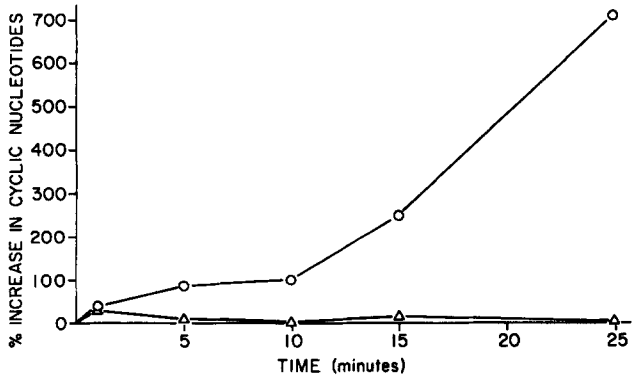

Figure 5. Effect of IBMX on gametic cAMP (○) and cGMP (△) content. Wild-type mt^+ gametes at 2 × 10^6/ml in 10 mM Pipes (pH 7.8) were exposed to a final concentration of 1 mM IBMX. At the indicated times, cells were pelleted and quenched with an equal volume of 6% TCA, and processed for cyclic nucleotides.

Table I. Gametic Responses to Candidate Signaling Agents

Cell type	Treatment	Cells displaying Wall loss	Cells displaying MSA	Cell type	Treatment	Cells displaying Wall loss	Cells displaying MSA
	mM	%				%	
mt$^+$	None	5	0		Ca^{++} + ionomycin		
	cAMP				0.2 mM + 5 μM	5	0
	10	6	1		1.0 mM + 5 μM	6	0
	50	13	3		Ca^{++} + ionomycin + dbcAMP + IBMX		
	db-cAMP				0.2 mM + 5 μM + 10 mM + 1 mM		
						80	50
	1	6(60)*	1(19)*		PMA		
	10	8	2		100 nM	6	0
	50	71	50		500 nM	5	0
	5′-AMP				Nigericin		
	10	7	0		10 μM	8	0
	50	6	0		Forskolin		
	Adenosine				100 μM	7	0
	10	4	0		H-8		
	50	7	0		0.5 mM	2	0
	IBMX				H-8 + db-cAMP + IBMX		
	0.5	10	3		0.5 mM + 10 mM + 1 mM	30	9
	1	48	15		1.0 mM + 10 mM + 1 mM	8	2
	db-cAMP + IBMX	19	9		db-cAMP + IBMX‡		
	1 + 0.5	14	7		10 mM + 1 mM	89	63
	10 + 0.5	36	17				
	10 + 1	95	68	Deflagellated	None	20	0
	Papaverine			*mt*$^+$§	db-cAMP + IBMX		
	0.05	83	8		10 mM + 1 mM	60	20
	0.1	100	13				
	db-cAMP + Papaverine			*bald-2 mt*$^+$§	None	4	0
	10 + 0.05	97	71		db-cAMP + IBMX		
	10 + 0.10	99	80		10 mM + 1 mM	52	15
	db-cGMP						
	20	6	0				
	db-cGMP + IBMX						
	20 + 1	23	8				
	8-Bromo-cGMP						
	5	6	0				
	10	7	0				
	8-Bromo-cGMP + IBMX						
	5 + 1	40	19				
	10 + 1	23	9				

Wild-type or mutant *mt*$^+$ gametes at 1 × 10^7 cell/ml in 10 mM Pipes (pH 7.8) were incubated at 23°C, with the indicated final concentration of agents, for 25 min unless otherwise indicated, and were then fixed by addition of an equal volume of 2% glutaraldehyde. Evaluation of cell wall loss and MSA by DIC was as described in Materials and Methods (Fig. 1). Since MSA cannot be detected in walled cells, nor when fertilization-tubule length is <0.5 μm, MSA is invariably scored less often than wall loss (but see Table II). Ionophores, phorbol myristate acetate (PMA), and forskolin stocks in DMSO were diluted such that the final DMSO concentration was <0.25%, a concentration which does not impair cell mating. Data are the averages of at least two experiments.

* Values in parenthesis resulted from a 2.5-h incubation with 1 mM dbcAMP.

‡ Cells pre-exposed to H-8 + db-cAMP + IBMX for 25 min were washed twice with Pipes and resuspended with db-cAMP + IBMX for an additional 25 min before fixation.

§ Cells were incubated for 60 min before fixation.

the singlet microtubules and the membrane; the singlets tend to look "filled"; and the singlets are elongated (not shown). Table II quantitates these responses for the two mating types.

The time course of gamete activation by exogenous db-cAMP (10 mM) + IBMX (1.0 mM) is shown in Fig. 9. Both wall loss and fertilization tubule activation show a lag phase that may be related to diffusion of the agents. Wall loss is essentially quantitative at 25 min. The failure of fertilization tubule activation to reach 100% may reflect, in part, the inability of the light microscope assay to reliably detect tubules <0.5-μm long; note in Table II that >90% activation is observed by electron microscopy. Interestingly, fertilization tubule activation could be reversed by washing cells out of the activating medium: 90% of cells had resorbed their tubules by 30 min post-wash (Fig. 9). Fertilization tubules activated by adhesion will similarly disassemble when the adhesion stimulus is removed (24). Cell wall resynthesis was not apparent by DIC during the 1-h post-wash observation period.

To determine whether flagella are required for cells to respond to exogenous db-cAMP, we studied the response of deflagellated cells and the flagella-less mutant *bald-2*. In both cases, db-cAMP and IBMX were still able to induce activation of wall loss and fertilization tubules (Fig. 1 *d* and Table I). However, the rate of response was slower than in

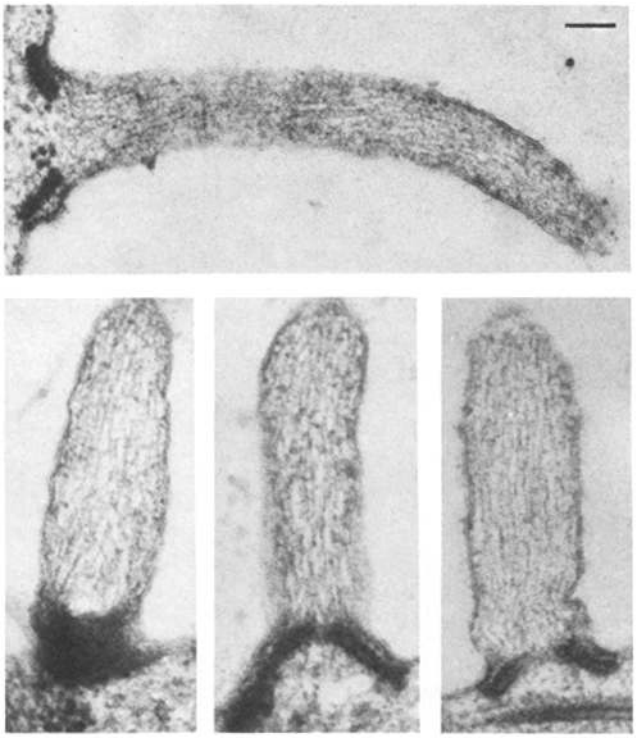

Figure 6. Fertilization tubules erected by mt^+ gametes in response to a 20-min exposure to 10 mM db-cAMP + 1 mM IBMX. Actin filaments extend from the dense mating structures towards the tips of the fertilization tubules (none of which are included in these sections). Bar, 100 nm.

flagellated cells: 60 min was required to produce levels of wall loss comparable to those seen after 25 min in flagellated cells (Table I). In such experiments, the values for fertilization tubule activation are likely to be underestimates since flagellaless cells do not rest on the slide with the horizontal preference displayed by flagellated cells, and mating structures are therefore not invariably in the plane of view.

Effects of Dibutyryl-cAMP + IBMX on Vegetative Cells

Gametes retain full-length, fully motile flagella after at least 4 h in db-cAMP + IBMX. This response is unlike that of vegetative (mitotic) cells, which have previously been shown to respond to IBMX by immediately becoming immotile and rapidly resorbing their flagella (36, 48). We confirmed this observation and found that IBMX-treated vegetative cells, unlike gametes, fail to shed their walls (mating-structure activation cannot be scored since vegetative cells lack mating structures). Importantly, the shortening vegetative flagella have a very different morphology from tip-activated gametic flagella. As shown in Fig. 8 (*bottom*), flagellar shortening entails axonemal disassembly, and the flagellum swells considerably as it fills with a flocculent material, presumably largely tubulin, which is less electron-dense than flagellar tip material (Fig. 8, *top*), and is randomly localized along the length of the axoneme. Although we cannot rule out the possibility that flagellar tip material as well as tubulin might be present in these disassembling flagella, the vegetative flagellar response to IBMX is clearly very different from the gametic response.

Analysis of Other Signaling Candidates

To assess whether other signaling agents might operate in addition to, or substitute for, the cAMP signal, a variety of agents were tested for their ability to induce cell wall loss or plus mating structure activation using DIC microscopy. Table I summarizes the data obtained with various agents that are known to act as elicitors or modifiers of signaled responses in other systems. Neither cell wall loss nor fertilization tubule activation was observed when plus gametes were treated with Ca^{++} plus ionophore (A23187 or ionomycin). That Ca^{++} was in fact entering the treated cells was evident by the fact that they rapidly swam to the bottom of the tube, consistent with the known alterations in phototaxis caused by increased intracellular calcium (45). The swimming response was not elicited by Ca^{++} or ionophore alone and was readily reversed by washing the cells free of ionophore. Table I also documents that no sexual responses were observed after treating plus gametes with nigericin with external pH clamped at 8.0 (30 mM Tris, 100 mM potassium gluconate, 1 mM $MgSO_4$), unlike responses reported with echinoderm sperm (74), nor were they observed after exposure to phorbol ester, monobutyryl 8-bromo-cGMP, dibutyryl-cGMP, or forskolin. Minus gametes treated with these reagents were similarly unresponsive with respect to wall loss; mating-structure activation in minus cells cannot be reliably scored by DIC.

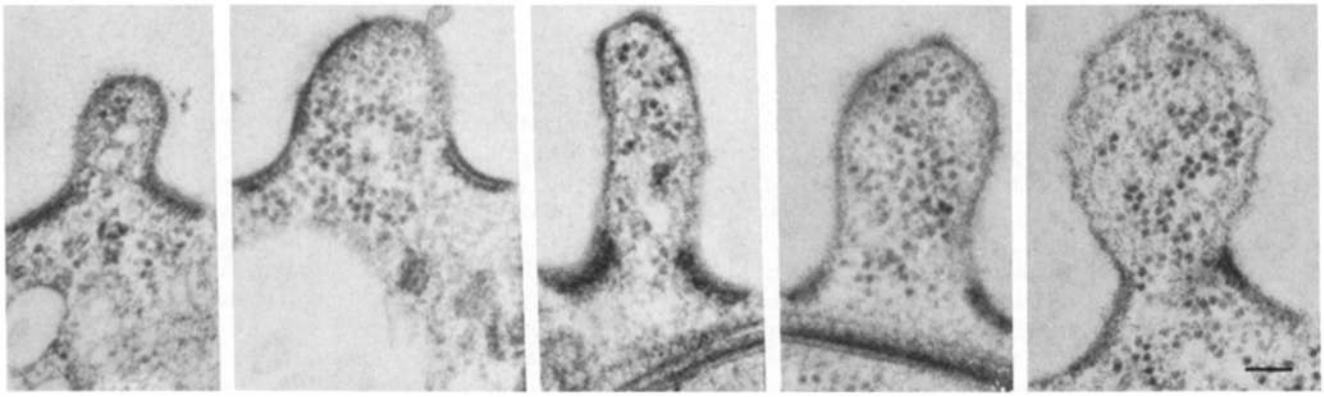

Figure 7. Activated mt^- mating structures in response to a 20-min exposure to 10 mM db-cAMP + 1 mM IBMX. Cytoplasmic swellings, usually containing ribosomes, extend from the dense "membrane zones" (32) of the mating structures. Bar, 100 nm.

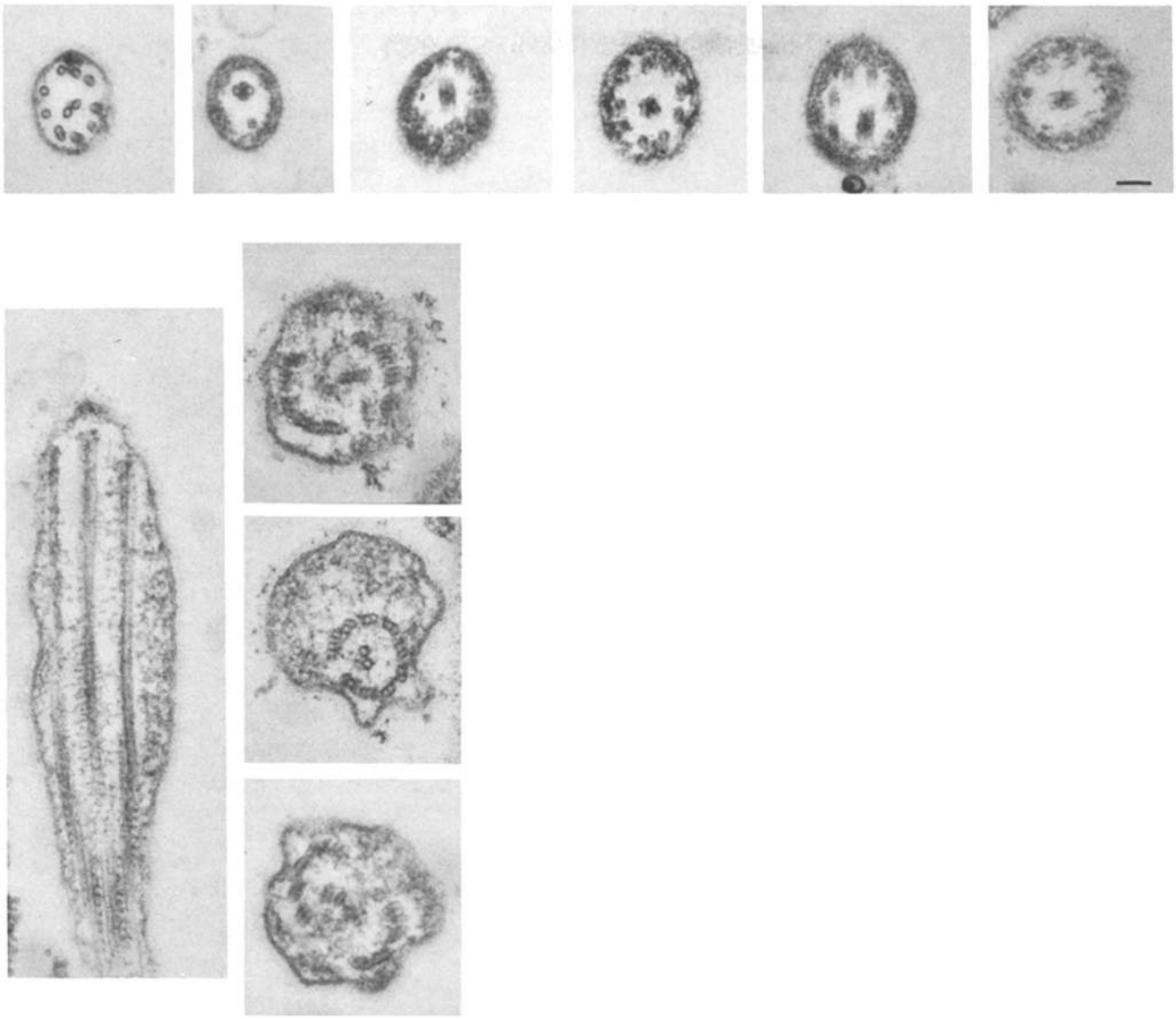

Figure 8. (*Top*) FTA induced by a 20-min exposure of gametes to 10 mM db-cAMP + 1 mM IBMX. Both mt^+ and mt^- gametic flagella are illustrated. The first flagellum to the left is not activated; its singlet microtubules are hollow, and only a small punctate plaque occupies the space between the microtubules and the membrane. The remaining flagella are activated: their microtubules appear "filled," and a continuous layer of dense fibrous tip material (53) lies between the microtubules and the membranes. (*Bottom*) Flagellar disassembly induced by a 15-min exposure of vegetative cells to 10 mM db-cAMP + 1 mM IBMX. The flagella become swollen and the axoneme loses its integrity. Flocculant material in the matrix compartment is less electron-dense than FTM and is not confined to the flagellar tips. Bar, 100 nm.

Mating Capability of Artificially Activated Gametes

If exogenous cAMP is mimicking the physiologic sexual signal and producing functionally, as well as morphologically, normal activation, then treated gametes should be primed to undergo cell fusion. We first established that activation by exogenous cAMP was not detrimental to mating by testing the ability of separately pre-activated plus and minus gametes to interact and fuse. Gametes treated with db-cAMP + IBMX were observed to agglutinate normally and, as shown in Table III, *a* and *b*, they were able to fuse at untreated control levels.

We next asked whether agglutinative flagellar interaction is a requirement for the fusion of cAMP-activated cells, as might be the case if agglutination generates an essential "fusion signal" that acts independently of cAMP production. We tested the requirement for agglutination by separately pre-activating two non-agglutinating mutants (*imp-2* mt^+ and *imp-l2* mt^-) and subsequently mixing them. As shown in Table III, *c* and *d*, pre-activation by db-cAMP + IBMX allowed 75% of the non-agglutinating population to fuse and become apparently normal quadriflagellated cells. In contrast, control *imp* cells, whose walls were removed by prior treatment with autolysin, fused at only 0.5%. Thus it appears that cAMP can activate cells for fusion independently of agglutination.

We also tested the ability of cAMP to promote fusion of cells that are artificially mating-blocked at points subsequent to agglutination. First we asked whether cAMP can rescue

Table II. Flagellar Tip Activation and Mating Structure Activation Induced by Elevated Levels of cAMP

	No. activated	No. non-activated	Activated
			%
Flagellar tips			
Plus gametes, IBMX	10	5	66
Plus gametes, IBMX/db-cAMP	29	8	78
Minus gametes, IBMX	23	7	77
Minus gametes, IBMX/db-cAMP	22	9	71
Mating structures			
Plus gametes, IBMX	18	2	90
Plus gametes, IBMX/db-cAMP	20	2	91
Minus gametes, IBMX	27	9	75
Minus gametes, IBMX/db-cAMP	10	4	71

Gametes at 1 × 10^7 cells/ml in nitrogen-free high-salt minimal medium were incubated for 25 min at 23°C with 1 mM IBMX with or without 10 mM db-cAMP. They were then fixed for transmission electron microscopy. Untreated control cells showed no flagellar tip activation or mating structure activation.

gametes blocked by TFP (reference 23 and Fig. 4). As shown in Table III *e*, plus and minus gametes separately exposed to TFP and then mixed were markedly impaired in mating, whereas cells exposed to TFP as above, and then to db-cAMP + IBMX in the presence of TFP, were able to mate at control levels (Table III, *f* and *g*). The mechanism of the TFP mating-block is unknown, but is presumed to be related to antagonism of calmodulin (CaM)-dependent step(s) required for sexual signal generation (23). The ability of cAMP to rescue TFP-blocked cells demonstrates that it bypasses this block and, together with the observation that TFP also impairs the normal rise of cAMP (Fig. 4), suggests that a cAMP elevation, perhaps CaM-mediated, is a necessary condition for the induction of mating events.

Second, we asked whether cAMP can rescue gametes mating-blocked by the protein kinase inhibitor H-8 (38). As shown in Table III *h*, plus and minus gametes pretreated with H-8 were prevented from mating; when observed by phase microscopy, they were found to agglutinate normally, but the adhesive foci failed to migrate to the flagellar tips and no cell wall loss occurred. Such H-8–blocked cells were not rescued by the addition of db-cAMP + IBMX (Table III *i*), implying that the locus of the H-8 effect is downstream of cAMP

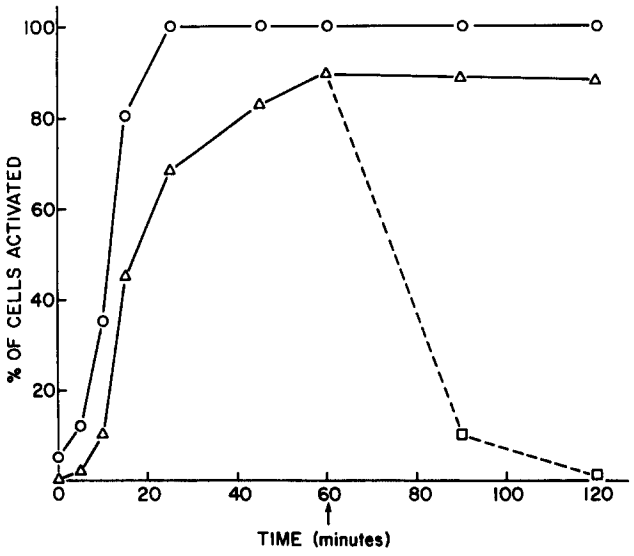

Figure 9. Kinetics of gamete activation by exogenous db-cAMP plus IBMX. Wild-type mt^+ gametes at 2 × 10^6/ml in 10 mM Pipes (pH 7.8) were exposed to a final concentration of 10 mM db-cAMP and 1 mM IBMX. At 60 min (*arrow*), half of the cells (□) were washed twice with 10 mM Pipes, and incubation was continued for an additional 60 min. At the indicated times, cells were fixed and scored for wall loss (○) and mating structure activation (△) by DIC.

Table III. Mating Responses of Artificially Activated Gametes

Mating	Cell Fusion
	%
$mt^+ \times mt^-$	
a. Control	90
b. db-cAMP + IBMX	88
imp-2 $mt^+ \times$ *imp-12* mt^-	
c. db-cAMP + IBMX	75
d. Autolysin	0.5
$mt^+ \times mt^-$	
e. TFP	13
f. TFP then db-cAMP + IBMX	81
g. Untreated control	89
$mt^+ \times mt^-$	
h. H-8	0.9
i. H-8 then db-cAMP + IBMX	1.5

a. Equal numbers of mt^+ and mt^- gametes at 1 × 10^7 cells/ml in 10 mM Pipes (pH 7.8) were mated for 20 min before fixation and quantitation of % cell fusion.

b. Gametes as above were treated separately with 10 mM db-cAMP + 1 mM IBMX for 20 min before mating.

c. Gametes at 4 × 10^7 cells/ml, separately pre-treated with 10 mM db-cAMP + 1 mM IBMX for 20 min, were then combined and incubated in the presence of db-cAMP + IBMX for an additional 40 min before fixation.

d. Gametes as above were treated with crude gametic autolysin for 20 min before mixing and incubation as above.

e. Gametes at 1 × 10^7 cells/ml were treated separately for 10 min with 4 μM TFP, then mixed and mated for 20 min before fixation.

f. Gametes were treated with TFP as above; however, 5 min after TFP addition, 10 mM db-cAMP + 1 mM IBMX was added and incubation continued for 5 more min before mating.

g. Untreated control mating.

h. Gametes at 1 × 10^7 cells/ml were treated separately with 500 μM H-8 for 5 min before mating.

i. Gametes were exposed to H-8 as above; however, 2.5 min after H-8 addition, 10 mM db-cAMP + 1 mM IBMX was added and incubation continued for an additional 2.5 min before mating.

Table IV. Fractionation of Gametic Adenylate Cyclase Activity

	Total protein	Specific activity	Total activity	Recovery of activity
	mg	*pmol/min per mg*	*pmol/min*	%
Whole cell sonicate	2,700	86	232,200	100
Cell body sonicate	1,890	55	98,917	42.6
17K *g* supernate	7.2	40	288	0.1
Detached flagella	1,125	64	71,936	31.0
Axonemes	900	8	7,200	3.1
Flagellar membrane vesicles	199	270	53,730	23.1
100K *g* supernate	6.0	50	300	0.1

Cell fractions prepared as described in Materials and Methods were assayed for adenylate cyclase activity. Sonicated samples were given two 15-s bursts using a Fisher model 300 microprobe demembranator at 0.5 relative output. In preliminary experiments, it was determined that sonication had no significant effect on cell body, flagellar, or vesicle fractions. Assay conditions were as described in Materials and Methods, with the exception that Mn^{++} was at 0.25 mM.

generation. This finding suggests that the effects of cAMP during mating are mediated, at least in part, by protein kinase-dependent phosphorylation events.

Flagellar Adenylate Cyclase and Phosphodiesterase

The results presented above suggest that during the natural mating reaction, sexual adhesion might stimulate an adenylate cyclase and/or inhibit a phosphodiesterase. Previous studies have demonstrated that adenylate cyclase (40) and cyclic nucleotide phosphodiesterase (25) activities are present in whole-cell extracts of vegetative *C. reinhardtii* cells, but to our knowledge, neither enzyme has been studied in *Chlamydomonas* gametes.

Table IV summarizes the distribution of adenylate cyclase activity in gametic cell fractions isolated and analyzed as described in Materials and Methods. Adenylate cyclase activity was present in both the cell body and flagellar fractions and showed linear reaction kinetics when a phosphodiesterase inhibitor (10 mM theophylline) was added to the reaction mixture (Fig. 10).

The flagellar membrane fraction was found to be highly enriched for cyclase activity (Fig. 11). No stimulation of activity was observed in the presence of 10 mM NaF (Fig. 12), whereas the enzyme was preferentially stimulated by Mn^{++} (Figs. 12 and 13). Fig. 13 presents the dose-response of the flagellar membrane adenylate cyclase to Mn^{++} and Mg^{++}. The response to Mn^{++} is biphasic, displaying a maximal stimulation of activity at ~0.5 mM. At this concentration, the ratio of Mn^{++}-stimulated to Mg^{++}-stimulated activity was 9:1.

To investigate the possibility of G protein (29) involvement, the flagellar membrane adenylate cyclase was assayed in the presence of agents known to activate G proteins (Table V). Neither GTP nor the nonhydrolyzable analogue GTPγS stimulated in vitro enzyme activity. AlF_4^-, an agent that binds to and activates G proteins (70), was likewise ineffective. Forskolin, an activator of the catalytic unit of G protein-coupled adenylate cyclase (22), also failed to stimulate the flagellar enzyme. Thus the data suggest that the enzyme is not associated with a stimulatory G protein. TFP was found to produce a dose-dependent inhibition of the flagellar membrane adenylate cyclase (Fig. 14), although the EC_{50} is higher than that found to inhibit the in vivo mating response (Fig. 4).

Cyclic nucleotide phosphodiesterase activity was studied in isolated flagella and was detected in the flagella of both

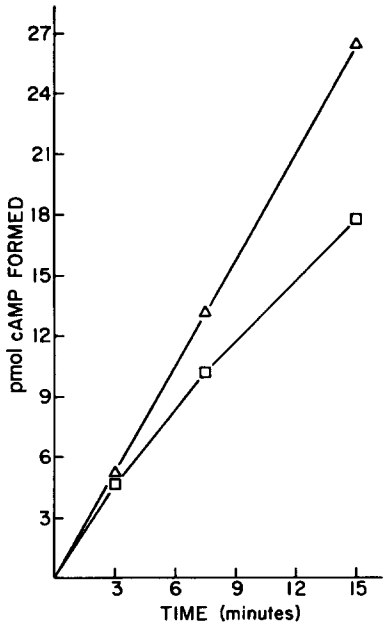

Figure 10. Effect of theophylline on adenylate cyclase activity in whole-cell sonicates of *C. reinhardtii*. Wild-type mt^+ gametes at 2 × 10^7/ml were sonicated. Theophylline-treated (10 mM, △) and control (□) samples (10-μg protein) were analyzed for adenylate cyclase activity in the presence of 5 mM Mg^{++}.

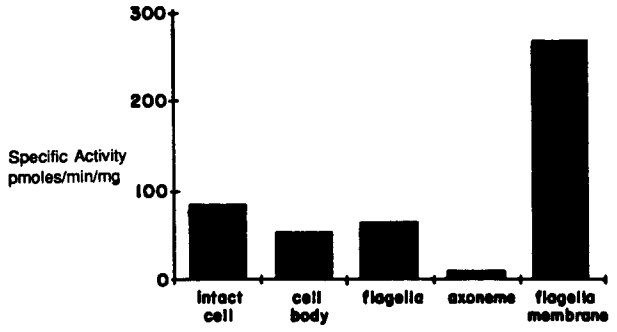

Figure 11. Distribution of adenylate cyclase activity in *C. reinhardtii* gametes. Cell fractions were analyzed for adenylate cyclase activity. Mn^{++} (0.25 mM) was included in the assay.

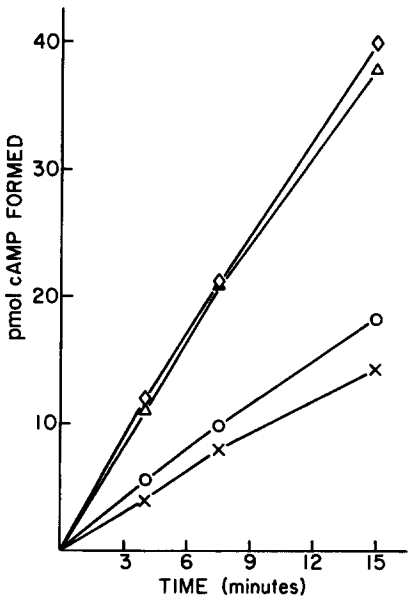

Figure 12. Effects of Mn^{++} and Mg^{++} on flagellar membrane adenylate cyclase in the presence and absence of NaF. Flagellar membrane vesicles (10 μg protein/assay) were assayed at 5 mM Mg^{++} (○), at 5 mM Mg^{++} + 10 mM NaF (×), at 5 mM Mn^{++} (△) and at 5 mM Mn^{++} + 10 mM NaF (◇).

plus and minus gametes (Table VI). IBMX effectively inhibited the flagellar enzyme (Table VI). The activity observed in flagella is lower by an order of magnitude than that previously reported for ammonium sulfate–fractionated extracts of whole vegetative cells (25); this may be due, in part, to poor penetration of the flagellar membrane by cAMP.

Discussion

cAMP Response during Mating

We present data describing a mating-associated elevation of cAMP in gametes of *C. reinhardtii* that parallels the response previously observed in mating gametes of *C. eugametos* (59). The kinetics of this response in a population of mating wild-type cells, returning to basal levels at the time that the slowest members of the population have completed mating,

Table V. Lack of Response of Flagellar Adenylate Cyclase to G Protein Activators or Forskolin

Agent	Specific activity
	pmol/min per mg
None (control)	172
GTP	
10 μM	171
100 μM	169
GTPγS	
10 μM	172
100 μM	148
AlF_4^-	
12.5 mM NaF + 12.0 μM Al^{3+}	182
25.0 mM NaF + 12.0 μM Al^{3+}	173
Forskolin	
10 μM	178
100 μM	175

Flagellar membranes were preincubated for 15 min at 37°C with various agents at the indicated concentrations. Adenylate cyclase reactions were initiated by addition of [^{32}P]ATP in the presence of 5 mM Mg^{2+}.

suggests that the response is initiated by mating-effective cell contact and terminated upon fusion of the cells, when flagella disagglutinate. The cAMP responses of mating-defective mutants support this interpretation. Failure of the non-agglutinating mutant *imp-5 mt*$^+$ to initiate a cAMP response when mixed with wild-type minus gametes indicates that an agglutinin-mediated cell–cell contact is required. The prolonged cAMP response during matings involving the nonfusing mutant *imp-1 mt*$^+$ implies that down-regulation of the cAMP response is dependent upon cell fusion or a post-fusion event. This prolonged cAMP response is unlike that of receptor/G protein–coupled mammalian cyclases, which are typically down-regulated rapidly in the continuing presence of activating ligand (17), and suggests that cAMP, or a labile cAMP-dependent product, may be continuously required during the mating process. Consistent with this interpretation is our observation that cAMP-induced fertilization tubule activation is reversed upon removal of agents that elevate internal cAMP levels (Fig. 9).

The basal cAMP levels detected in *C. reinhardtii* (10 ± 2 pmol/10^8 cells) are higher by an order of magnitude than

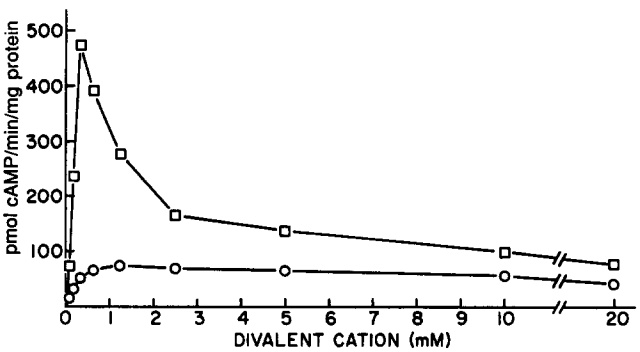

Figure 13. Dose-response of flagellar membrane adenylate cyclase to Mn^{++} (□) and Mg^{++} (○). Flagellar membrane vesicles were at 10 μg protein/assay.

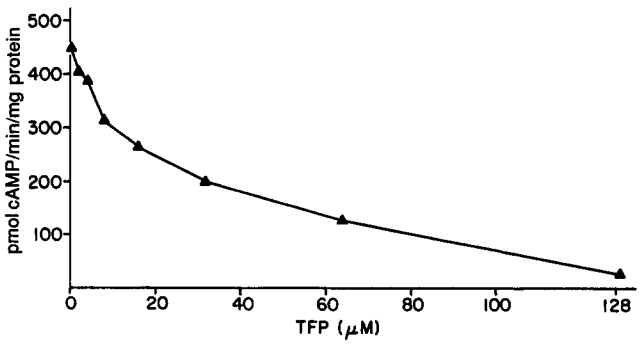

Figure 14. Effect of TFP on the in vitro activity of flagellar membrane adenylate cyclase. Flagellar membrane vesicles (10 μg protein/assay) were incubated for 5 min with the indicated concentrations of TFP before cAMP assay.

Table VI. Cyclic Nucleotide Phosphodiesterase Activity of Detached Gametic Flagella

	pmol cAMP consumed/min per mg
mt^+ Flagella	12.5
mt^+ Flagella + IBMX	0.01
mt^- Flagella	20.1
mt^- Flagella + IBMX	0.02

Detached flagella (50 μg) were analyzed for cyclic nucleotide phosphodiesterase in both the presence and absence of 1 mM IBMX.

those reported by Pijst et al. (59) for *C. eugametos*. They are, on the other hand, comparable to those reported for a wide variety of gametes from *Limulus* to human (27). Likewise, the extent of cAMP increase during mating is threefold lower than that observed for *C. eugametos* (59), but is again very similar to that observed in sperm cells responding to egg-associated factors (28).

Activation of Mating Events by cAMP

As summarized in Tables I–III, db-cAMP, either acting alone or synergistically with phosphodiesterase inhibitors, induces tipping, flagellar tip activation, cell wall loss, mating structure activation, and readiness for cell fusion in gametes of both mating types. This is the first demonstration that cAMP can function as a sufficient initial signal of mating events in *Chlamydomonas*. It is also the first demonstration that mating responses can be elicited by a stimulus other than adhesion. The most unexpected observation in this regard is the occurrence of tipping and FTA in non-adherent cells in response to cAMP elevation. We had previously assumed (33, 53) that both flagellar responses were associated with adhesion per se. Instead, it appears that agglutination causes a rise in intraflagellar cAMP which, directly or via secondary effectors, brings about the altered flagellar surface motility and tip morphology. Although the biological function of FTA is not known, previous investigators (21, 53) have suggested that it might cause, or at least promote, the near-immotile state of gametes engaged in adhesion. Since cAMP-activated gametes display normal flagellar motility, this idea is also refuted by the present study.

The ability of methylxanthine and non-methylxanthine agents to inhibit a flagellar cyclic nucleotide phosphodiesterase activity (Table VI) and to elevate endogenous cAMP levels (Fig. 5), as well as induce morphologically typical mating reaction events in synergy with db-cAMP (Table I), argues that the phosphodiesterase inhibitor effects are in fact cAMP-related and not nonspecific.

Cell Type Specificity of the cAMP Response

The *Chlamydomonas* life cycle includes three differentiated cell types: the haploid vegetative cell capable of mitosis, the haploid gamete capable of fertilization, and the diploid zygote capable of long-term dormancy and meiosis. In keeping with the role of a second messenger, cAMP elicits distinctive responses in different *C. reinhardtii* cell types. Specifically, the mating events induced in gametes by IBMX are very different from the effects of IBMX on vegetative cells. As first reported by others (36, 48, 60), methylxanthine phosphodiesterase inhibitors cause vegetative cells to become immotile and to resorb their flagella, whereas gametes remain fully motile for hours in the presence of IBMX. Moreover, the movement of microspheres along the vegetative flagellar surface is inhibited by IBMX (9), whereas in gametes the microspheres are induced by IBMX to move to the flagellar tips. Regeneration of vegetative flagella is also prevented by these agents. Although the biological basis of the vegetative response to methylxanthines is unknown, it is of interest that in *Chlamydomonas*, the first visible sign that a cell is entering mitotic prophase is rapid flagellar resorption (43). Since mitotic prophase in vertebrate cells and *Physarum* is heralded by phosphorylation of specific proteins (52), and methylxanthines have been shown to induce early onset of division (62), a rise in cAMP levels may be of general importance in driving cells into mitosis.

Flagellar Membrane Adenylate Cyclase

Adenylate cyclase activity has previously been reported in whole-cell sonicates of *C. reinhardtii* vegetative cells (40). Here we report the first determinations of adenylate cyclase in gametes of this species. Further, we have established that the enzyme is present in both cell body and flagellar fractions, with flagellar membranes possessing the highest specific activity of the fractions studied.

Adenylate cyclase activity has been described in association with ciliary and flagellar membranes in several species, where it is thought to participate in sensory transducing mechanisms (e.g., olfactory transduction [58]), and motility regulation in response to environmental factors (64). The ciliary membrane adenylate cyclases of lower eukaryotes (75), as well as the enzyme from molluscan (47) and mammalian (39) sperm, appear to share several properties that differentiate them from the receptor–G protein coupled adenylate cyclases of mammals. Specifically, the enzymes are not stimulated in vitro by guanine nucleotides, fluoride, cholera toxin, or forskolin, and they frequently display high ratios of Mn-stimulated to Mg-stimulated activity. The results reported in this study indicate that the flagellar membrane adenylate cyclase of *C. reinhardtii* gametes also shares these properties. Although our data indicate that a classic stimulatory G protein does not operate in this system, such negative functional data must be interpreted with caution in view of the fact that in both *Dictyostelium* (49) and spermatozoa (6), similar negative functional data have recently been called into question by the demonstration of membrane species that can be ribosylated with cholera toxin plus NAD^+.

The possibility that cAMP, acting as a sexual signal, is produced by a CaM-regulated adenylate cyclase is suggested by the following observations: CaM has been identified in *Chlamydomonas* flagella (57); the CaM antagonist TFP inhibits all post-adhesion mating events (23) and also inhibits the cAMP response in agglutinating cells (Fig. 3); the mating-block induced by TFP can be by-passed by exogenous cAMP (Table III); and TFP inhibits the in vitro activity of flagellar membrane adenylate cyclase (Fig. 15). Further studies of CaM regulation of adenylate cyclase are in progress.

The presence of adenylate cyclase and cyclic nucleotide phosphodiesterase activities in flagella suggests that sexual adhesion may modulate the activity of one or both enzymes, either directly or via some agent such as CaM. However, this

remains to be demonstrated. In preliminary experiments, we were unable to detect changes in the activity of either enzyme during the in vitro interaction of detached plus and minus flagella (30), but since detached flagella display functional abnormalities – they neither tip nor inactivate their agglutinin molecules – their failure to transduce the sexual signal may well be an in vitro artifact. Flagella per se are clearly not necessary for wall loss and mating structure activation, as demonstrated by the mating responses of deflagellated and *bald-2* flagellaless mutants provided with db-cAMP plus IBMX (Fig. 1 *d* and Table I). Since the response to IBMX by these flagellaless gametes is relatively slow, it could be argued that they lack a major source of cAMP, that is, that the flagellum contains most of the cell's adenylate cyclase activity. However, we have not yet ruled out the possibility that the flagellar membranes are simply a highly permeable avenue through which drugs can enter the cell, and that the agglutination-associated modulation of cAMP is in fact effected by non-flagellar enzymes.

cAMP as a Physiologic Sexual Signal in Chlamydomonas

The present study has met the four criteria originally proposed by Sutherland et al. (72) for the establishment of cAMP as a physiologic second messenger. Specifically, an increase in the endogenous level of cAMP was demonstrated during the mating response, addition of exogenous cAMP triggered mating events, cyclic nucleotide phosphodiesterase inhibitors enhanced the effects of exogenous cAMP while increasing the level of endogenous cAMP, and adenylate cyclase activity was demonstrated in a plasma membrane fraction of the cell. Taken together, these findings provide strong evidence that cAMP is a primary physiologic signal during mating in *Chlamydomonas*. Further support for this conclusion is provided by the finding that cAMP is required continuously for the maintenance of fertilization tubule activation, and that non-agglutinating cAMP-activated cells mate normally.

The candidacy of cAMP as a primary signal messenger during *Chlamydomonas* mating is also strengthened by the fact that other candidate signaling agents give no evidence of being operative. cGMP analogues are unable to signal on their own and actually suppress the effects of IBMX when the two are administered together (Table I). The magnitude and kinetics of the cGMP response during mating likewise suggest that it is not a primary sexual signal: cGMP levels show only a twofold increase during mating, and fail to return to basal levels subsequent to disadhesion (Fig. 2). Such a pattern could conceivably indicate a role for cGMP in effecting a "turn off" of the mating response, but there is no reason to believe that cGMP plays any role in triggering the response. We also show that cytoplasmic alkalinization, which elicits actin polymerization in echinoderm sperm (74) and eggs (5), is without effect in *Chlamydomonas*; it is also without effect on actin polymerization in neutrophils (56). Phorbol esters also failed to elicit mating responses in *Chlamydomonas*, discouraging the hypothesis that adhesion stimulates protein kinase C–dependent phosphorylation events as in certain other systems (79). Finally, the failure of calcium plus ionophore to induce mating events, or to function synergistically with cAMP, argues against a role for calcium as the primary sexual signal. Evidence for mating-associated calcium fluxes has, however, been reported (10, 46, 68). Indeed, one study reports direct activation of wall loss in both vegetative cells and gametes by calcium plus A23187 (16), a result we could not reproduce. That Ca^{++} alone cannot elicit sexual responses does not, of course, rule out a role for Ca^{++} in regulating early responses to adhesion, a role suggested by the experiments of Detmers and Condeelis (23) and by our finding that the flagellar adenylate cyclase is inhibited by the calmodulin antagonist TFP.

The mechanism by which cAMP might function to effect tipping, FTA, wall loss, and mating structure activation is unknown. cAMP-dependent protein kinase activity has been demonstrated in gametes of *Chlamydomonas eugametos* (59). To date, however, there has been no investigation as to whether unique cAMP-dependent phosphorylation events are triggered by mating in *Chlamydomonas*. Our finding that H-8, an inhibitor of cyclic nucleotide–dependent protein kinase (38), inhibits mating and reversibly antagonizes the responses of gametes to exogenous cAMP, suggests that at least one of the sexual signaling functions of cAMP may involve activation of protein kinases. An alternative, non-enzymatic mechanism by which cAMP might be functioning in *Chlamydomonas* is analogous to the role of cGMP in photoreceptor cells (71), which are ciliary derivatives. Here, cGMP binds directly to ion channels to maintain them in an open conformation and promote ion fluxes. Such a role for cAMP has recently been reported in olfactory receptor cilia membranes (55); its applicability to the *Chlamydomonas* system remains to be addressed.

The similarity between the actin-filled fertilization tubule of *C. reinhardtii* gametes and the actin-filled acrosome of echinoderm sperm (74) has previously been noted (32). The present study establishes a second common feature of the two gamete types: both respond to sexual adhesion with an 8–10-fold transient increase in intracellular levels of cAMP (28). Thus a common evolutionary thread may link *Chlamydomonas* with the germ line of the lower eukaryotic metazoa.

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