Flagellar Adhesion-dependent Regulation of *Chlamydomonas* Adenylyl Cyclase In Vitro: A Possible Role for Protein Kinases in Sexual Signaling

Yuhua Zhang and William J. Snell

Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical School, Dallas, Texas 75235

Abstract. Interactions between adhesion molecules, agglutinins, on the surfaces of the flagella of mt^+ and mt^- gametes in *Chlamydomonas* rapidly generate a sexual signal, mediated by cAMP, that prepares the cells for fusion to form a zygote. The mechanism that couples agglutinin interactions to increased cellular levels of cAMP is unknown. In previous studies on the adenylyl cyclase in flagella of a single mating type (i.e., non-adhering flagella) we presented evidence that the gametic form of the enzyme, but not the vegetative form, was regulated by phosphorylation and dephosphorylation (Zhang, Y., E. M. Ross, and W. J. Snell. 1991. *J. Biol. Chem.* 266:22954–22959; Zhang, Y., and W. J. Snell. 1993. *J. Biol. Chem.* 268:1786–1791). In the present report we describe studies on regulation of flagellar adenylyl cyclase during adhesion in a cell-free system. The results show that the activity of gametic flagellar adenylyl cyclase is regulated by adhesion in vitro between flagella isolated from mt^+ and mt^- gametes. After mixing mt^+ and mt^- flagella together for 15 s in vitro, adenylyl cyclase activity was increased two- to threefold compared to that of the non-mixed (non-adhering), control flagella. This indicates that the regulation of gametic flagellar adenylyl cyclase during the early steps of fertilization is not mediated by signals from the cell body, but is a direct and primary response to interactions between mt^+ and mt^- agglutinins.

By use of this in vitro assay, we discovered that 50 nM staurosporine (a protein kinase inhibitor) blocked adhesion-induced activation of adenylyl cyclase in vitro, while it had no effect on adenylyl cyclase activity of non-adhering gametic flagella. This same low concentration of staurosporine also inhibited adhesion-induced increases in vivo in cellular cAMP and blocked subsequent cellular responses to adhesion. Taken together, our results indicate that flagellar adenylyl cyclase in *Chlamydomonas* gametes is coupled to interactions between mt^+ and mt^- agglutinins by a staurosporine-sensitive activity, probably a protein kinase.

ALTHOUGH one result of an interaction between two developmentally primed cells can be the formation of a stable cell-to-cell adhesion, an equally important consequence often is the generation of a cellular signal. Cell contact-induced cellular signaling has been shown to be important during fertilization (Snell, 1990), in development of the nervous system (Kapfhammer and Schwab, 1992), in eye development in *Drosophila* (Hart et al., 1993), and in the immune system (Hynes, 1992; Dustin and Springer, 1991). During fertilization, cell–cell contacts between sperm and egg initiate G protein signaled responses in both cell types (Jaffe, 1990; Kopf, 1990; Ward and Kopf, 1993). Although several molecules involved in cell–cell adhesion have been identified, the molecular mechanisms coupling adhesion to signal transduction are only beginning to be understood. Presumably, cell–cell signaling involves molecular events similar to those that occur during more fully characterized receptor–ligand interactions in which the receptor is an integral membrane protein and the ligand is soluble. In cell–cell signaling, however, both of the interacting molecules are on the cell surface. During development of the compound eye in *Drosophila*, for example, interactions of the receptor tyrosine kinase, sevenless, on an R7 photoreceptor cell with its transmembrane ligand, bride of sevenless, on an adjacent R8 photoreceptor cell are required for normal retinal development (Hart et al., 1993).

Our laboratory has been interested in identifying the molecules responsible for signal transduction induced by cell contact during fertilization in the biflagellated alga, *Chlamydomonas reinhardtii*. Upon mixing, mt^+ and mt^- gametes of *Chlamydomonas* adhere to each other via adhesion molecules, agglutinins, on the surfaces of their flagella. Flagellar

Address all correspondence to W. J. Snell, Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical School, Dallas, TX 75235.

adhesion is followed by a rapid increase of intracellular cAMP (Pijst et al., 1984; Pasquale and Goodenough, 1987; Kooijman et al., 1990) leading to several cellular responses required for continued adhesion as well as for preparation of the interacting cells for fusion and zygote formation (Goodenough, 1989; Snell et al., 1989; Kooijman et al., 1989; Tomson et al., 1990). This specific, cell–cell adhesion and signaling system is unique to gametes, and many of the signaled responses that occur in the cell body can be induced by incubation of gametes of a single mating type in dibutyryl cAMP (db-CAMP)[1] (Pasquale and Goodenough, 1987; Snell et al., 1989; Goodenough, 1989; Hunnicutt et al., 1990). Vegetatively growing cells, on the other hand, do not express agglutinins and are unresponsive to incubation with db-cAMP (for reviews see Goodenough, 1991; van den Ende, 1992; and Snell, 1993).

With the goal of identifying and characterizing molecules in this signaling pathway, recently we began to study the adenylyl cyclase in *Chlamydomonas* flagella. Work from other laboratories (Pasquale and Goodenough, 1987; Kooijman et al., 1990) as well as our own (Zhang et al., 1991; Zhang and Snell, 1993) has suggested that, like the enzyme in sea urchin and mammalian sperm (Hildebrandt et al., 1985; Bookbinder et al., 1990), this adenylyl cyclase is not regulated by G proteins. Rather, our results are consistent with the idea that the adenylyl cyclase in gametic flagella is regulated by phosphorylation and dephosphorylation. The gametic flagellar adenylyl cyclase is inhibited by prior incubation of flagella with ATP and activity re-appears if the flagella are washed out of the ATP and incubated at room temperature in ATP-free buffer (Zhang et al., 1991). In more recent work we found that the ATP-dependent regulatory mechanism was unique to the adenyl cyclase of gametic flagella and was not present in vegetative flagella (Zhang and Snell, 1993), results that since have been confirmed by Saito et al. (1993). The existence of novel, gamete-specific mechanisms for regulating the flagellar adenylyl cyclase suggested that this enzyme might play a role in signal transduction induced by adhesion.

One model for this signaling pathway in gametes is that the events induced by agglutinin interactions, including the initial generation of cAMP, occur in the flagella. Consistent with this idea, Saito et al. (1993) showed that the adenylyl cyclase in flagella isolated from mt^+ and mt^- gametes that had been adhering for 3 min was increased twofold over flagella isolated from non-adhering gametes. Although these data support the model that activation of flagellar adenylyl cyclase is a primary event in signal transduction induced by agglutinin interactions, the results are indirect and other interpretations cannot be ruled out. Gametes respond to adhesion rapidly, and within seconds to minutes the cell bodies undergo changes involving Ca^{2+} utilization (Snell et al., 1982; Bloodgood and Levin, 1983; Kaska et al., 1985; Schuring et al., 1990; Goodenough et al., 1993) and changes in cAMP levels. The responses in cell bodies to these signals include secretion of a serine protease, loss of the cell wall, erection of an actin-filled mating structure, and movement of agglutinin molecules from the cell body onto the flagella (reviewed in Snell, 1993). Thus, is it possible that the increase in flagellar adenylyl cyclase shown by Saito et al. (1993) was not a primary effect of flagellar adhesion, but was one of the many responses to adhesion-induced signals from the cell body.

To examine this adhesion-induced signaling pathway directly we have begun to study the effects of flagellar adhesion on flagellar adenylyl cyclase activity in a cell-free system. In this report we show that the adenylyl cyclase activity of gametic flagella was activated nearly threefold simply by mixing isolated mt^+ and mt^- flagella together in vitro. Activation of adenylyl cyclase was detected within 15 s after mixing, indicating that regulation of this enzyme is a direct and immediate consequence of flagellar agglutinin interactions. Furthermore, we discovered that adhesion-induced activation was blocked by 50 nM staurosporine, a protein kinase inhibitor. Significantly, this low concentration of staurosporine also blocked adhesion-induced signaling in vivo. Our results indicate that interaction between agglutinins on flagella of opposite mating types rapidly activates flagellar adenylyl cyclase, probably via a multi-step pathway. This may be a novel example of regulation of this enzyme by membrane–membrane interactions in a cell-free system.

Materials and Methods

Materials

Hepes was from Research Organics Inc. (Cleveland, OH); pyruvate kinase (PK) was from Boehringer Mannheim GmbH (Mannheim, FRG); [^{3}H]cAMP was from ICN Biomedicals Inc. (Boston, MA; dimethyl sulfoxide was from J. T. Baker, Inc. (Phillipsburg, NJ); H-7 was from Calbiochem-Behring Corp. (Indianapolis, IN); H-8 was from Seikagaku Kogyo Co. (Tokyo, Japan); all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cells and Cell Culture

Chlamydomonas reinhardtii strains 21gr (mt^+) and 614c (mt^-) (available from the *Chlamydomonas* Genetics Center, Duke University, Durham, NC) were cultured at room temperature in medium I or medium II of Sager and Granick (1954; Harris, 1989) on a 13–11-h light–dark cycle and gametic cells were obtained as previously described (Snell, 1976).

Isolation of Flagella

Vegetative and gametic flagella were harvested by a modification of the pH shock method of Witman et al. (1972) as described earlier (Zhang et al., 1991). The sedimented flagella were resuspended to give a final concentration of 3–8 mg/ml flagellar protein in flagella buffer, which was 20 mM Na-Hepes, pH 7.2, 4% sucrose, 1 mM EDTA, 0.5% BSA, 2.5 mM $MgCl_2$, 0.05 mM GTP, 0.1 mM papaverine or 0.1 mM R020-1724. A mixture of protease inhibitors (7 μM leupeptin, 3.2 μg/ml trypsin inhibitor from lima bean, 60 μM *N*-tosyl-L-phenylalanine chloromethylketone (TPCK), 60 μM *N*-tosyl-L-lysine chloromethylketone (TLCK), and 0.13 mM PMSF) was added to the suspension of flagella; samples were stored in small aliquots in liquid N_2.

Adhesion In Vitro Between mt^+ and mt^- Flagella

Equal volumes of freshly isolated mt^+ and mt^- flagella were mixed together on ice in flagella buffer for 15 s and immediately frozen in liquid N_2. In experiments with protein kinase or protein phosphatase inhibitors, the inhibitors were added before the flagella were mixed.

Assay for Loss of Cell Walls

Cells that have lost their walls during the mating reaction become sensitive

1. *Abbreviations used in this paper*: App(NH)p, 5'-adenylylimidodiphosphate; db-cAMP, dibutyryl cAMP; PEP, phosphoenolpyruvate; PK, pyruvate kinase; TLCK, *N*-tosyl-L-lysine chloromethylketone; TPCK, *N*-tosyl-L-phenylalanine chloromethylketone.

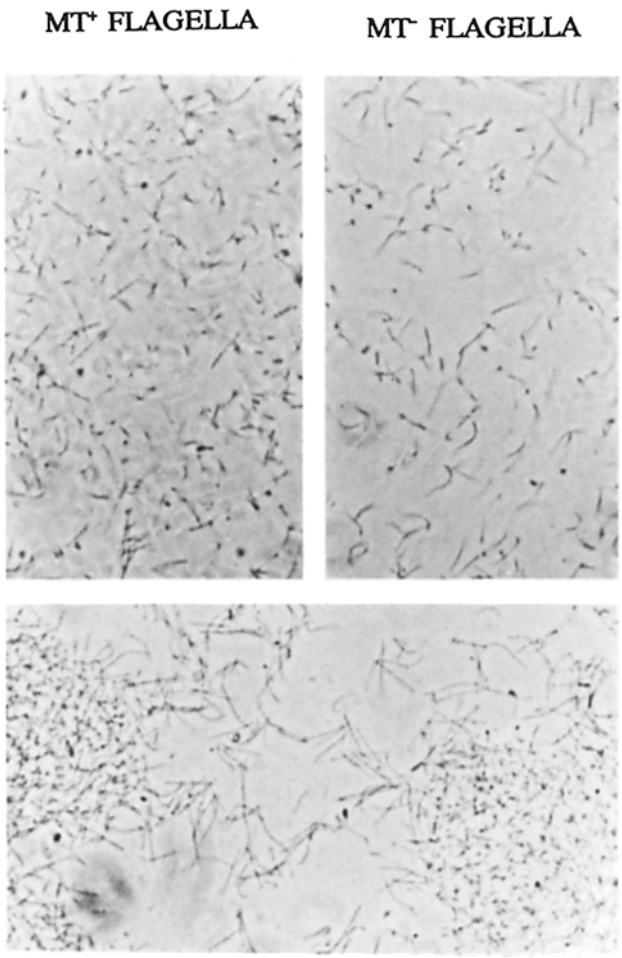

Figure 1. In vitro adhesion of gametic mt$^+$ and mt$^-$ flagella. The top left panel is a phase-contrast micrograph of freshly isolated mt$^+$ gametic flagella and the top right panel is of freshly isolated mt$^-$ gametic flagella before mixing. The bottom panel is a micrograph of a mixture of freshly isolated mt$^+$ and mt$^-$ flagella.

to lysis by detergent and release their chlorophyll, which can be detected spectrophotometrically at 435 nm. To carry out the assay 100 μl of cells at a density of 0.5–1 $\times$ 10^7 cells/ml was mixed with 500 μl of detergent solution containing 0.075% Triton X-100 and 10 mM EDTA. The sample was vortexed briefly, non-disrupted cells were sedimented by centrifugation in a microfuge for 15 s, and the OD_{435} of the supernatant was determined. Results shown are the averages of duplicate samples.

Protein Determination

Protein was determined with the Coomassie blue protein assay reagent of Pierce Chemical Co. (Rockford, IL) using crystalline BSA as standard.

Adenylyl Cyclase Assay

Adenylyl cyclase activity was measured essentially according to the methods of Ross et al. (1977) as described earlier (Zhang et al., 1991). Results shown are the averages of duplicate samples, which usually varied by less than 10%.

cAMP Assay

Relative cellular cAMP levels were determined based on the method described by Salomon (1991). Mt$^+$ gametes (1–5 $\times$ 10^7 cells/ml in N-free medium) were preincubated in [^{3}H]adenine (10 μCi/ml) at room temperature with aeration for 2 h. After preincubation cells were washed once with N-free medium to remove unincorporated [^{3}H]adenine. To determine changes in intracellular cAMP level during mating, labeled mt$^+$ gametes were mixed with non-labeled mt$^-$ gametes for varying times, and the reaction was terminated by addition of an equal volume of stop solution (10% TCA containing 0.2 mM cAMP). Samples were kept at 4°C for 30 min with occasional vortexing to extract nucleotides, centrifuged at 3,000 *g* for 5 min and the supernatants were loaded onto Dowex 50 and alumina columns to separate cAMP from ATP as described for the adenylyl cyclase assay above. Recovery of cAMP, typically about 50%, was determined by comparing the OD_{259} of the samples before and after chromatography.

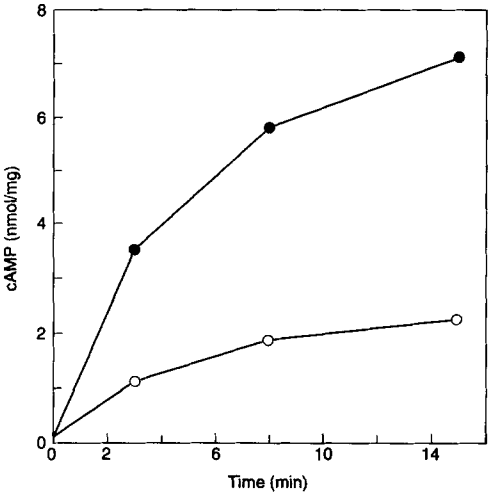

Figure 2. Adhesion-induced activation of adenylyl cyclase. Freshly isolated mt$^+$ and mt$^-$ gametic flagella were mixed on ice in buffer containing 20 mM Na-Hepes, pH 7.2, 4% sucrose, 1 mM EDTA, 0.5% BSA, 2.5 mM $MgCl_2$, and 0.1 mM RO20-1724. After 15 s the samples were quick-frozen in liquid N_2. After thawing, adenylyl cyclase assays were performed at 30°C for the indicated times. The adenylyl cyclase activity in the mixture of mt$^+$ and mt$^-$ flagella (●) and the average of the activities of non-mixed mt$^+$ and mt$^-$ flagella (○) are plotted against the indicated assay times. 55 μg of mt$^+$ flagellar protein and 50 μg of mt$^-$ flagellar protein were used in the assays.

Results

Adhesion-induced Activation of Flagellar Adenylyl Cyclase In Vitro

As originally described by Kohle et al. (1980) and confirmed by Goodenough (1986) flagella isolated from mt$^+$ and mt$^-$ gametes adhered avidly to each other via their flagellar agglutinins and formed large aggregates when mixed together in vitro (Fig. 1, *bottom*). The upper panels in Fig. 1 show that there was no adhesion within samples of flagella isolated from gametes of a single mating type (mt$^+$, *upper left*; mt$^-$, *upper right*). To determine if in vitro adhesion was coupled to changes in adenylyl cyclase activity, freshly isolated flagella were mixed together and then assayed for adenylyl cyclase activity. Fig. 2 shows that the flagella that had been mixed (*closed circles*) exhibited increased adenylyl cyclase activity compared to the average of the non-mixed mt$^+$ and mt$^-$ flagella (*open circles*). At the 15-min point of the assay the amount of cAMP formed by the mixed samples was threefold greater than the average of the non-mixed samples. This activation of adenyl cyclase required mixing of freshly

Table I. Effect of Protein Kinase and Protein Phosphatase Inhibitors on Adhesion-induced Activation of Adenylyl Cyclase

Treatment	Adenylyl cyclase activity		Inhibition of activation
	Non-mixed	Mixed	
	pmol/mg/min		%
Control	230	575	0
Calyculin A (1 μM)	241	583	5
Okadaic acid (1 μM)	244	538	19
NaF (10 mM)	271	608	17
Vanadate (0.1 mM)	274	642	11
H-8 (1 mM)	258	610	9
H-7 (1 mM)	235	581	2
Staurosporine (50 nM)	237	348	69

Freshly isolated mt^+ and mt^- gametic flagella were mixed in the presence of the inhibitor for 15 s, quick-frozen, and then assayed at 30°C for 8 min as described in Fig. 2. Assays contained 120 μg of mt^+ flagellar protein and 98 μg of mt^- flagellar protein.

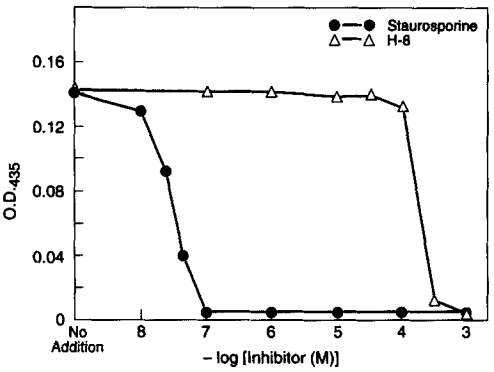

Figure 4. Effect of staurosporine and H-8 cell signaling during mating. Mt^+ and mt^- gametes (2×10^7 cells/ml) were pretreated with H-8 and staurosporine at the indicated concentrations at room temperature for 30 min. Equal volumes of gametes of opposite mating types then were mixed together and wall loss was determined as described in Materials and Methods. In the control, non-treated samples about 90% of the cells lost their walls.

isolated, gametic flagella. Mixing of freshly isolated vegetative flagella of opposite mating types did not lead to activation of adenylyl cyclase and no increase in adenylyl cyclase activity was detected if gametic flagella of opposite mating types were frozen and thawed before mixing (data not shown). These results indicated that activation of flagellar adenylyl cyclase does not require signals from the cell body, but is a direct consequence of interactions between agglutinins on mt^+ and mt^- flagella.

Regulation of Adhesion-induced Activation of Adenylyl Cyclase by Staurosporine

To learn about possible regulatory events in adhesion-induced activation of adenylyl cyclase we tested the effects of inhibitors of protein kinases and protein phosphatases. Several protein phosphatase inhibitors including calyculin A, okadaic acid, NaF and vanadate had little or no effect on adhesion-induced activation (Table I). Similarly, the protein kinase inhibitors H-8 and H-7 were without effect on adhesion-induced activation of flagellar adenylyl cyclase (Table I). On the other hand 50 nM staurosporine blocked about 70% of the activation of the enzyme in adhering samples, while the activity in non-mixed samples was unaffected by this drug treatment (Table I). These results suggested that a protein kinase was required at an early step in adhesion-induced regulation of adenylyl cyclase.

This inhibition of adhesion-induced activation by staurosporine was somewhat surprising because earlier experiments from our laboratory showed that gametic adenylyl cyclase in isolated flagella of a single mating type (i.e., non-adhering flagella) was stimulated by staurosporine (Zhang et al., 1991; Zhang and Snell, 1993). Our earlier experiments, however, were done with 1 μM staurosporine, whereas the experiments reported in Table I were performed with 50 nM staurosporine. To examine further the sensitivity to staurosporine of the adenylyl cyclase in non-adhering flagella, we determined the effects of varying concentrations of this inhibitor on adenylyl cyclase activity in flagella of a single mating type. As shown in Fig. 3, the stimulation of adenylyl cyclase in non-adhering flagella occurred only at high concentrations (500 nM and above) of inhibitor and stimulation was not detectable at 50 nM. The results in Fig. 3 and Table I confirmed our earlier report of a 1 μM staurosporine-sensitive regulation of adenylyl cyclase in non-adhering flagella, but also indicated that a second protein kinase activity, which was sensitive to 50 nM staurosporine, acted at an early step in the signaling pathway that coupled interactions between mt^+ and mt^- agglutinins to activation of flagellar adenylyl cyclase.

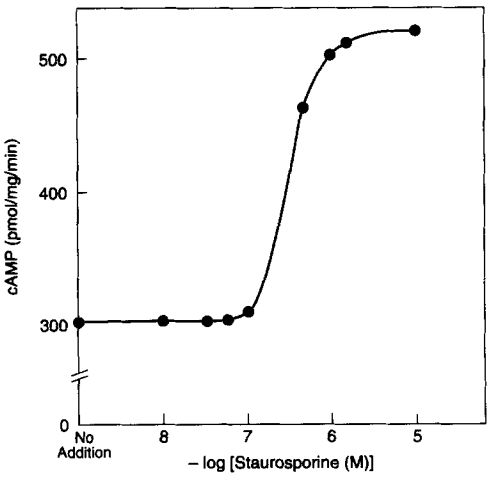

Figure 3. Dose dependence of activation of non-mixed flagella by staurosporine. The adenylyl cyclase activity of isolated gametic flagella was assayed as described in Materials and Methods in the indicated concentrations of staurosporine at 30°C for 10 min. The amount of protein of each assay was 90 μg.

Staurosporine Inhibition of Adhesion-induced Signaling In Vivo and Rescue of Signaling by Dibutyryl cAMP

To evaluate the effects of protein kinase inhibitors on signaling in vivo we mixed mt^+ and mt^- gametes together in the presence of varying amounts of H-8 or staurosporine and

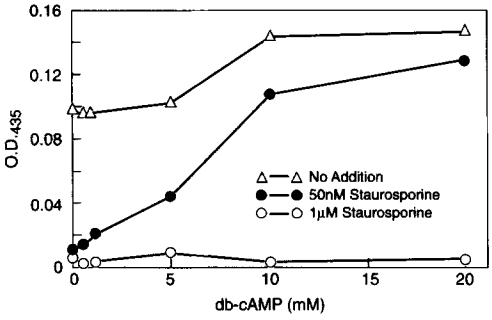

Figure 5. Rescue of the staurosporine-induced inhibition of signaling by db-cAMP. 1 ml of mt^+ and mt^- gametes (2×10^7 cells/ml) were pretreated without (△) or with 50 nM (●) or 1 μM staurosporine (○) at room temperature for 30 min. At the end of the incubation, 150 μl of mt^+ cells were mixed with the same volume of mt^- cells for 10 min at room temperature and then db-cAMP was added to yield the indicated concentrations. All samples contained 0.1 mM papaverine. Samples were incubated for another 45 min at room temperature with shaking. Subsequently, 180 μl portions were mixed with 520 μl of the detergent solution described in Materials and Methods. The OD_{435} of the control cells treated with 20 mM db-cAMP represented about 85% wall loss.

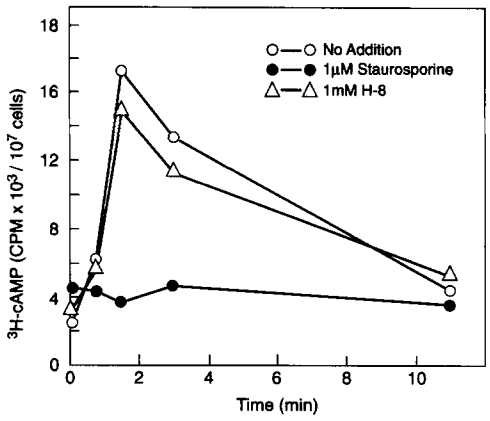

Figure 6. Effect of staurosporine on intracellular cAMP levels during the mating reaction. 12 ml of mt^+ gametes (5×10^7 cells per ml in N-free medium) were incubated with [^{3}H]adenine as described in Materials and Methods and pretreated without (○) or with 1 μM staurosporine (●) or 1 mM H-8 (△) at room temperature for 30 min. Subsequently, 250-μl portions of these radiolabeled, staurosporine-treated mt^+ gametes were mixed with 250-μl portions of non-treated or staurosporine-treated mt^- gametes for the times indicated. The reactions were terminated by addition of 500 μl of stop solution and relative levels of cAMP were determined as described in Materials and Methods.

then determined the extent of cell wall loss as a measure of cell signaling. Confirming earlier reports (Pasquale and Goodenough, 1987; Goodenough, 1993), 0.5–1 mM H-8 substantially inhibited wall loss (Fig. 4, *triangles*). This inhibition of signaled events by H-8 in vivo at first might seem to be inconsistent with the lack of effect of H-8 in vitro on adenylyl cyclase activity reported earlier (Zhang et al., 1991) and also shown in Table I. But Pasquale and Goodenough (1987) and Goodenough (1993) showed that H-8 blocked at a late step in signaling in vivo because the inhibition by H-8 could not be overcome by addition of db-cAMP, results also confirmed by us (data not shown).

Staurosporine, on the other hand, inhibited wall loss at low concentrations (Fig. 4, *circles*), half-maximal inhibition being observed at about 50 nM. This low concentration was similar to the concentration that produced nearly 70% inhibition of adhesion-induced activation of adenylyl cyclase in vitro (Table I). Also unlike the results with H-8, the inhibition of wall loss by 50 nM staurosporine could be rescued by addition of db-cAMP as shown in Fig. 5. In this experiment mt^+ and mt^- gametes were separately incubated with buffer alone (Fig. 5, *triangles*) or with 50 nM (*closed circles*) or 1 μM staurosporine (*open circles*) for 30 min at room temperature. The pretreated gametes of opposite mating types were mixed together for 10 min and then the indicated concentrations of db-cAMP (and papaverine) were added. After 45 min the samples were assayed for wall loss, which was measured as the appearance of chlorophyll (OD_{435}) in supernatants of detergent-treated cells as previously described (Snell, 1980; Buchanan and Snell, 1988). In the absence of db-cAMP, cell wall loss and cell fusion (data not shown) were blocked by both 50 nm and 1 μM staurosporine.

db-cAMP and papaverine had little effect on wall loss in the control cells not treated with staurosporine (Fig. 5, *open triangles*), although 10 mM and higher concentration of db-cAMP gave some increase of wall release. In addition db-cAMP did not rescue signaling in cells that were pretreated with 1 μM staurosporine (Fig. 5, *open circles*). Even at 20 mM db-cAMP no release of cell walls occurred. This inability to rescue wall loss in gametes in 1 μm staurosporine indicated that at high concentrations this inhibitor was blocking a late step in signaling. In contrast, however, db-cAMP rescued signaling in cells pretreated with 50 nM staurosporine (Fig. 5, *closed circles*). At 5 mM db-cAMP wall loss reached about 40% of control levels and at 20 mM db-cAMP wall loss was nearly 90% of controls. Cells treated with db-cAMP also recovered their ability to undergo cell fusion (data not shown).

Although these results were consistent with an effect by staurosporine on a step before (upstream of) activation of adenylyl cyclase, we wanted to determine more directly if adenylyl cyclase activity was blocked by staurosporine and not affected by H-8, a putative downstream inhibitor. To do this we measured changes in cellular cAMP levels at various times during the mating reaction in the presence and absence of H-8 or staurosporine. Confirming earlier reports by Pijst et al. (1984) and Pasquale and Goodenough (1987) there was a rapid, 8–10-fold increase of cellular cAMP within 2 min after non-pretreated, control mt^+ and mt^- gametes were mixed together (Fig. 6, *open circles*). Cells pretreated with 1 mM H-8 and then mixed together in the continued presence of the inhibitor showed a similar increase in cAMP (Fig. 6, *open triangles*), indicating that although H-8 blocked late stages of signaling (Fig. 4), this inhibitor had no effect on the initial events in this signaling pathway.

In cells pretreated with 1 μM staurosporine (Fig. 6, *closed circles*), however, we obtained different results. Although this treatment led to nearly a twofold increase in basal levels of cAMP, the adhesion-induced increase of intracellular cAMP was blocked completely. Similar results were obtained with 100 nM staurosporine (data not shown). Thus,

both the in vivo and in vitro results were consistent with the idea that a staurosporine-sensitive protein kinase acts early in adhesion-induced signaling, at a step in the pathway that couples agglutinin interaction to activation of adenylyl cyclase.

Discussion

Adhesion-induced Activation of Flagellar Adenylyl Cyclase in a Cell-free System

In this report we have shown that adhesion in vitro between isolated mt^+ and mt^- gametic flagella of *Chlamydomonas* activated the flagellar adenylyl cyclase severalfold (Fig. 2). While soluble ligands regulate adenylyl cyclase activity in other systems, we believe that this is a novel demonstration of activation of this signal-transducing enzyme by interactions between endogenous, membrane-bound, cell adhesion molecules in a cell-free system. The data shown here provide direct evidence that the flagellar adenylyl cyclase is one site of regulation of cAMP levels during adhesion. In our experiments the increase in adenylyl cyclase occurred within 15 s after flagella were mixed together, indicating that adhesion-induced activation was a direct and primary response to agglutinin interactions and did not require signals from the cell body.

Because the agglutinin may not be a transmembrane protein (reviewed in Adair, 1985), coupling of agglutinin interactions to the adenylyl cyclase could require intermediate transmembrane components (Kooijman et al., 1989; van den Ende, 1992). This observation, along with the evidence cited above suggesting that G proteins do not play a role in this adhesion-induced signal transduction pathway, has compelled us to search for a novel mechanism of regulation of adenylyl cyclase in *Chlamydomonas*. Previously we presented evidence that gametic flagella from non-adhering cells contain a novel, ATP-dependent mechanism for regulation of adenylyl cyclase. Our use of this cell-free system for activation of adenylyl cyclase has permitted us to search for an adhesion-dependent mechanism that regulates adenylyl cyclase during fertilization in *Chlamydomonas*.

A Possible Role for a Protein Kinase in the Pathway Coupling Agglutinin Interactions to Activation of Flagellar Adenylyl Cyclase

By use of this in vitro system we discovered a new step in the signal transduction pathway between agglutinin interactions and activation of adenylyl cyclase. The results in Table I show that the adhesion-dependent activation of flagellar adenylyl cyclase in vitro was inhibited by low concentrations (50 nM) of the protein kinase inhibitor staurosporine. This suggests that interactions between mt^+ and mt^- agglutinins activate a protein kinase that is required for activation of adenylyl cyclase. This *Chlamydomonas* signaling pathway is not the only one in which protein kinases have been implicated in regulating adenylyl cyclase. Nair and Patel (1993) have reported that the tyrosine kinase activity of the epidermal growth factor receptor is essential for stimulation of cardiac adenylyl cyclase by epidermal growth factor. And recent work from several laboratories has indicated that mammalian adenylyl cyclases are regulated by protein kinase C, a staurosporine-sensitive protein kinase, although it is not known if the adenylyl cyclase itself is phosphorylated in these systems (Jacobowitz et al., 1992; Yoshimura and Cooper, 1993; Lustig et al., 1993; Frings, 1993; Choi et al., 1993).

Our previous studies (Zhang et al., 1991; Zhang and Snell, 1993) on gametic flagella of a single mating type (i.e., non-adhering flagella) indicated that they contain an ATP-dependent inhibitor of the flagellar adenylyl cyclase. This inhibitor had the properties of a heat-labile, 1 μM staurosporine-sensitive protein kinase whose action constitutively inhibited the adenylyl cyclase in non-adhering flagella. The results shown in Fig. 3 indicated that this putative protein kinase was not inhibited by the low concentration of staurosporine (50 nm) that blocked the adhesion-induced activation of adenylyl cyclase in vitro (Table I).

The in vitro results that 50 nm staurosporine inhibited adhesion-induced activation of adenylyl cyclase were confirmed by the in vivo studies shown in Fig. 4. Low concentrations of staurosporine blocked signal transduction when mt^+ and mt^- gametes were mixed together; inhibitor-treated cells did not undergo cell fusion (not shown) nor did they undergo cell wall loss, one of the cellular responses to increased levels of intracellular cAMP.

A Staurosporine-sensitive, Early Step during Signaling In Vivo

Our results on inhibition of adhesion-induced events by staurosporine are consistent with data reported earlier by other workers. Goodenough (1993) showed that 1 μM staurosporine inhibited cell fusion. These workers, however, did not determine the effects of staurosporine on the adhesion-induced increase in cAMP. Since the block to cell fusion could not be rescued by db-cAMP, they concluded that this inhibitor acted primarily on "downstream" events (Goodenough, 1993; Saito et al., 1993).

We confirmed that the 1 μM staurosporine inhibition of adhesion-induced events could not be rescued by db-cAMP, but we also found that the inhibition by 50 nM staurosporine could be rescued by db-cAMP (Fig. 5). These results are consistent with the idea that at least two steps in the signaling pathway are blocked by this inhibitor. An early step, adhesion-induced activation of adenylyl cyclase, is inhibited both by high and low concentrations of staurosporine; whereas a later step, which requires cAMP, is unaffected by low concentrations of this inhibitor, but is blocked by 1 μM staurosporine. Similar to the effect of 1 μM staurosporine and as reported earlier (Pasquale and Goodenough, 1987; Goodenough, 1993) another protein kinase inhibitor, H-8, also blocked cell wall loss and other signaled events and could not be rescued by db-cAMP. In contrast to staurosporine, however, H-8 had no effect on the adhesion-induced increase in cAMP (Fig. 6), indicating that H-8 acts only on later steps in the pathway.

A Working Model for Regulation of Flagellar Adenylyl Cyclase in Gametes

Chlamydomonas gametes are faced with the not uncommon problem of using a single second messenger, in this case cAMP, for more than one regulatory function. The motility of flagella of non-adhering gametes and vegetative cells (Pasquale and Goodenough, 1988; Hasegawa et al., 1987) as well as the motility of flagella and cilia from several other

species (reviewed in Tash, 1989; and Stephens and Stommel, 1989) can be regulated by cAMP. But *Chlamydomonas* also uses this cyclic nucleotide as a signaling molecule during flagellar adhesion between gametes, when cAMP levels increase 8–10-fold over normal levels. Our evidence suggests that gametic flagella accomplish this exquisite, dual control of cAMP levels in gametes by regulating the activity of flagellar adenylyl cyclase. Data from this and previous reports are consistent with the following working model for regulation of gametic flagellar adenylyl cyclase. In nonadhering gametes, flagellar adenylyl cyclase activity is kept at a low level by the action of a protein kinase that is sensitive to 1 μM staurosporine. The presence of this putative, inhibitory protein kinase was indicated by the twofold increase in the activity of adenylyl cyclase resulting from incubating gametes (Fig. 6) or isolated flagella (Fig. 3) in 1 μM staurosporine. Full activation of the flagellar adenylyl cyclase during flagellar adhesion, however, requires the activation of a different protein kinase that can be inhibited by 50 nM staurosporine. This newly described activator of adenylyl cyclase is stimulated by interactions between mt^+ and mt^- flagellar agglutinins both in vivo (Fig. 6) and in vitro (Fig. 2, Table I). The relationship between the adhesion-dependent activator of adenylyl cyclase and the constitutive, ATP-dependent inhibitor of adenylyl cyclase is unclear. The adhesion-dependent activator could regulate the constitutive, ATP-dependent inhibitor of adenylyl cyclase, or it could act directly on the adenylyl cyclase. Further investigations of these putative protein kinases will be required to understand the molecular mechanisms underlying this signal transduction pathway.

By analogy with receptors for growth hormones or cytokines (Davis et al., 1993; Murakami et al., 1993), it may be that binding of agglutinins induces aggregation of an agglutinin–anchor protein complex (Kooijman et al., 1989; Kalshoven et al., 1990; Bloodgood and Salomonsky, 1991), thereby initiating the signaling pathway. Saito et al. (1993) have shown that lectins and anti-flagellar antibodies, which should cross-link flagellar surface molecules, can activate the flagellar adenylyl cyclase about twofold. These workers and Goodenough et al. (1993) have proposed that adhesion-induced activation of the flagellar adenylyl cyclase requires both this twofold enhancement as well as Ca^{2+}-dependent stimulation. Further studies should reveal the relationship, if any, between this putative Ca^{2+}-dependent activator proposed by these other workers and the 50 nM staurosporine sensitive, adhesion-dependent activator described in this report.

The authors would like to thank Dr. Fred Grinnell for helpful discussions and for reading the manuscript.

This work was supported by National Institutes of Health grant GM25661 to W. J. Snell.

Received for publication 9 December 1993 and in revised form 4 February 1994.

References

Adair, W. S. 1985. Characterization of *Chlamydomonas* sexual agglutinins. *J. Cell Sci. Suppl.* 2:233–260.

Bloodgood, R. A., and E. N. Levin. 1983. Transient increase in calcium efflux accompanies fertilization in *Chlamydomonas reinhardii*. *J. Cell Biol.* 97:397–404.

Bloodgood, R. A., and N. L. Salomonsky. 1991. Regulation of flagellar glycoprotien movements by protein phosphorylation. *Eur. J. Cell Biol.* 54:85–89.

Bookbinder, L. H., G. W. Moy, and V. D. Vacquier. 1990. Identification of sea urchin sperm adenylate cyclase. *J. Cell Biol.* 111:1859–1866.

Choi, E. J., S. T. Wong, A. H. Dittman, and D. R. Storm. 1993. Phorbol ester stimulation of the type-I and type-III adenylyl cyclases in whole cells. *Biochemistry.* 32:1891–1894.

Davis, S., T. H. Aldrich, N. Stahl, L. Pan, T. Taga, T. Kishimoto, N. Y. Ip, and G. D. Yancopoulos. 1993. LIFRβ and gp 130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science (Wash. DC).* 260: 1805–1808.

Dustin, M. L., and T. A. Springer. 1991. Role of lymphocyte adhesion receptors in transient interactions and cell locomotion. *Annu. Rev. Immunol.* 9:27–66.

Frings, S. 1993. Protein kinase C sensitizes olfactory adenylate cyclase. *J. Gen. Physiol.* 101:183–205.

Goodenough, U. W. 1986. Experimental analysis of the adhesion reaction between isolated *Chlamydomonas* flagella. *Exp. Cell Res.* 166:237–246.

Goodenough, U. W. 1989. Cyclic AMP enhances the sexual agglutinability of *Chlamydomonas* flagella. *J. Cell Biol.* 109:247–252.

Goodenough, U. W. 1993. Tipping of flagellar agglutinins by gametes of *Chlamydomonas reinhardtii*. *Cell Motil. & Cytoskeleton.* 25:179–189.

Goodenough, U. W., W. S. Adair, B. Caligor, C. L. Forest, J. L. Hoffman, D. A. Mesland, and S. Speth. 1980. Membrane-membrane and membrane-ligand interactions in *Chlamydomonas* mating. *In* Membrane-Membrane Interactions. N. B. Gilula, editor. Raven Press, New York. 131–152.

Goodenough, U. W., B. Shames, L. Small, T. Saito, R. C. Crain, M. A. Sanders, and J. L. Salisbury. 1993. The role of calcium in the *Chlamydomonas reinhardtii* mating reaction. *J. Cell Biol.* 121:365–374.

Harris, E. H. 1989. The *Chlamydomonas* Sourcebook: A Comprehensive Guide to Biology and Laboratory Use. Academic Press, San Diego. 780 pp.

Hart, A. C., H. Kramer, and S. L. Zipursky. 1993. Extracellular domain of the boss transmembrane ligand acts as an antagonist of the sev receptor. *Nature (Lond.).* 361:732–736.

Hasegawa, E., H. Hayashi, S. Asakura, and R. Kamiya. 1987. Stimulation of in vitro motility of *Chlamydomonas* axonemes by inhibition of cAMP-dependent phosphorylation. *Cell Motil. & Cytoskel.* 8:302–311.

Hildebrandt, J. D., J. Codina, J. S. Tash, H. J. Kirchick, L. Lipschultz, R. D. Sekura, and L. Birnbaumer. 1985. The membrane-bound spermatozoal adenylyl cyclase system does not share coupling characteristics with somatic cell adenylyl cyclases. *Endocrinology.* 116:1357–1366.

Hunnicutt, G. R., M. G. Kosfiszer, and W. J. Snell. 1990. Cell body and flagellar agglutinins in *Chlamydomonas reinhardtii*: the cell body plasma membrane is a reservoir for agglutinins whose migration to the flagella is regulated by a functional barrier. *J. Cell Biol.* 111:1605–1616.

Hynes, R. O. 1992. Integrins—versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11–25.

Jacobowitz, O., J. Chen, R. T. Premont, and R. Iyengar. 1993. Stimulation of specific types of Gs-stimulated adenylyl cyclases by phorbol ester treatment. *J. Biol. Chem.* 268:3829–3832.

Jaffe, L. A. 1990. First messengers at fertilization. *J. Reprod. Fert.* 42 (Suppl.):107–116.

Kalshoven, H. W., A. Musgrave, and H. van den Ende. 1990. Mating receptor complex in the flagellar membrane of *Chlamydomonas eugametos* gametes. *Sex. Plant Reprod.* 3:77–87.

Kapfhammer, J. P., and M. E. Schwab. 1992. Modulators of neuronal migration and neurite growth. *Curr. Opin. Cell Biol.* 4:863–868.

Kaska, D. D., I. C. Piscopo, and A. Gibor. 1985. Intracellular calcium redistribution during mating in *Chlamydomonas reinhardtii*. *Exp. Cell. Res.* 160: 371–379.

Kohle, D., W. Lang, and H. Kauss. 1980. Agglutination and glycosyltransferase activity of isolated gametic flagella from *Chlamydomonas reinhardtii*. *Arch. Microbiol.* 127:239–243.

Kooijman, R., P. de Wildt, S. Beumer, G. van der Vliet, W. Homan, H. Kalshoven, A. Musgrave, and H. van den Ende. 1989. Wheat germ agglutinin induces mating reactions in *Chlamydomonas eugametos* by cross-linking agglutinin-associated glycoproteins in the flagellar membrane. *J. Cell Biol.* 109:1677–1687.

Kooijman, R., P. de Wildt, W. van den Briel, A. Musgrave, and H. van den Ende. 1990. Cyclic AMP is one of the intracellular signals during the mating of *Chlamydomonas eugametos*. *Planta.* 181:529–537.

Kopf, G. S. 1990. Zona pellucida-mediated signal transduction in mammalian spermatozoa. *J. Reprod. Fert.* 42(Suppl.):33–49.

Lustig, K. D., B. R. Conklin, P. Herzmark, R. Taussig, and H. R. Bourne. 1993. Type I adenylylcyclase integrates coincident signals from Gs, Gi, and Gq. *J. Biol. Chem.* 268:13900–13905.

Murakami, M., M. Hibi, N. Nakagawa, T. Nakagawa, K. Yasukawa, K. Yamanishi, T. Taga, and T. Kishimoto. 1993. *Science (Wash. DC).* 260: 1808–1810.

Nair, B. G., and T. B. Patel. 1993. Regulation of cardiac adenylyl cyclase by epidermal growth factor (EGF)—role of EGF receptor protein tyrosine kinase activity. *Biochem. Pharmacol.* 46:1239–1245.

Pasquale, S. M., and U. W. Goodenough. 1987. Cyclic AMP functions as a primary sexual signal in gametes of *Chlamydomonas reinhardtii*. *J. Cell Biol.* 105:2279–2292.

Pasquale, S. M., and U. W. Goodenough. 1988. Calmodulin sensitivity of the flagellar membrane adenylate cyclase and signalling of motile responses by cAMP in gametes of *Chlamydomonas reinhardtii*. *Botanica Acta.* 101: 118–122.

Pijst, H. L. A., R. van Driel, P. M. W. Janssens, A. Musgrave, and H. van den Ende. 1984. Cyclic AMP is involved in sexual reproduction of *Chlamydomonas eugametos*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 174: 132–136.

Ross, E. M., M. E. Maguire, T. W. Sturgill, R. L. Biltonen, and A. G. Gilman. 1977. Relationship between the beta-adrenergic receptor and adenylate cyclase. *J. Biol. Chem.* 252:5761–5775.

Sager, R., and S. Granick. 1954. Nutritional control of sexuality in *Chlamydomonas reinhardtii*. *J. Gen. Physiol.* 37:729–742.

Saito, T., Y. Tsubo, and Y. Matsuda. 1985. Synthesis and turnover of cell body agglutinin as a pool of flagellar surface agglutinin in *Chlamydomonas reinhardtii* gamete. *Arch. Microbiol.* 142:207–210.

Saito, T., L. Small, and U. W. Goodenough. 1993. Activation of adenylyl cyclase in *Chlamydomonas reinhardtii* by adhesion and by heat. *J. Cell Biol.* 122:137–147.

Salomon, Y. 1991. Cellular responsiveness to hormones and neurotransmitters: conversion of [^{3}H]-Adenine to [^{3}H]-cAMP in cell monolayers, cell suspensions, and tissue slices. *Methods Enzymol.* 195:22–28.

Schuring, F., J. Brederoo, A. Musgrave, and H. van den Ende. 1990. Increase in calcium triggers mating structure activation in *Chlamydomonas eugametos*. *Fed. Eur. Microbiol. Soc.* 71:237–240.

Snell, W. J. 1976. Mating in *Chlamydomonas*: a system for the study of specific cell adhesion. I. Ultrastructural and electrophoretic analysis of flagellar surface components involved in adhesion. *J. Cell Biol.* 68:48–69.

Snell, W. J. 1990. Adhesion and signaling in multicellular and unicellular organisms. *Curr. Opin. Cell Biol.* 2:821–832.

Snell, W. J. 1993. Signal transduction during fertilization in *Chlamydomonas*. *In* Signal Transduction: Procaryotic and Simple Eucaryotic Systems. J. Kurjan, editor. Academic Press, New York. 255–277.

Snell, W. J., M. Buchanan, and A. Clausell. 1982. Lidocaine reversibly inhibits fertilization in *Chlamydomonas*: a possible role for calcium in sexual signalling. *J. Cell Biol.* 94:607–612.

Snell, W. J., A. Clausell, and W. S. Moore. 1983. Flagellar adhesion in *Chlamydomonas* induces synthesis of two high molecular weight cell surface proteins. *J. Cell Biol.* 96:589–597.

Snell, W. J., W. A. Eskue, and M. J. Buchanan. 1989. Regulated secretion of a serine protease that activates an extracellular matrix-degrading metalloprotease during fertilization in *Chlamydomonas*. *J. Cell Biol.* 109:1689–1694.

Tash, J. S. 1989. Protein phosphorylation: the second messenger signal transducer of flagellar motility. *Cell Motil. Cytoskeleton.* 14:332–339.

Tomson, A. M., R. Demets, E. A. van Spronsen, G. J. Brakenhoff, D. Stegwee, and H. van den Ende. 1990. Turnover and transport of agglutinins in conjugating *Chlamydomonas* gametes. *Protoplasma.* 155:200–209.

van den Ende, H. 1992. Sexual signalling in *Chlamydomonas*. *In* Cellular Recognition. J. A. Callow and J. R. Green, editors. Cambridge University Press, Cambridge. 1–19.

Ward, C. R., and G. S. Kopf. 1993. Molecular events mediating sperm activation. *Dev. Biol.* 158:9–34.

Witman, G. B., K. Carlson, J. Berliner, and J. L. Rosenbaum. 1972. *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *J. Cell Biol.* 54:507–539.

Yoshimura, M., and D. M. F. Cooper. 1993. Type-specific stimulation of adenylylcylase by protein kinase C. *J. Biol. Chem.* 268:4604–4607.

Zhang, Y., and W. J. Snell. 1993. Differential regulation of adenylylcyclases in vegetative and gametic flagella of *Chlamydomonas*. *J. Biol. Chem.* 268: 1786–1791.

Zhang, Y., E. M. Ross, and W. J. Snell. 1991. ATP-dependent regulation of flagellar adenylylcyclase in gametes of *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 266:22954–22959.