Cyclic 3′,5′-AMP Relay in *Dictyostelium discoideum*: Adaptation Is Independent of Activation of Adenylate Cyclase

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ABSTRACT In *Dictyostelium discoideum*, binding of cAMP to high affinity surface receptors leads to a rapid activation of adenylate cyclase followed by subsequent adaptation within several minutes. The rate of secretion of [^{3}H]cAMP, which reflects the state of activation of the enzyme, was measured. Caffeine noncompetitively inhibited the response to cAMP. Inhibition was rapidly reversible and pretreatment of cells with caffeine for up to 22 min had little effect on the subsequent responsiveness to cAMP. However, cells pretreated with caffeine plus cAMP for ≥8 min did not respond when caffeine was removed and the same concentration of cAMP was applied. The following observations indicate that both adaptation and deadaptation to cAMP occurred to the same extent and at the same rate whether or not cAMP synthesis was inhibited. First, when cells were pretreated with 10^{-9}–10^{-6} M cAMP in the presence or absence of caffeine and the stimulus was switched to a saturating dose of cAMP, the response to the increment was the same whether or not the initial response was blocked. Second, cells progressively lost responsiveness to 10^{-6} M cAMP as pretreatment with 10^{-6} M cAMP plus caffeine was extended from 0 to 8 min with the same time course as for those pretreated with 10^{-6} M cAMP alone. Third, cells which were adapted in the presence of caffeine and cAMP deadapted within the same time period as controls when cAMP was removed. These observations demonstrate that while some part of the activation process is inhibited by caffeine the adaptation process is unaffected. Our conclusion is that adaptation does not depend on the activation of adenylate cyclase.

During the developmental cycle of *Dictyostelium discoideum* individual amebas aggregate to form a multicellular organism containing ~10^5–10^6 cells. cAMP, acting extracellularly as a hormone, directs the aggregation. Periodically secreted by central cells, the chemoattractant diffuses to nearby cells, binds to high affinity surface receptors, and transiently activates adenylate cyclase. The newly synthesized cAMP is secreted and triggers the same response in more distal cells. This cell-to-cell relay of the chemical signal can be observed as cAMP waves, which propagate through the cell monolayer and provide gradients that guide the chemotactically sensitive cells toward the central emitting sources (1–3).

The cAMP-stimulated activation of adenylate cyclase, the resulting increase in intracellular cAMP, and cAMP secretion are referred to as the cAMP signaling response. There are two key properties of the cAMP signaling response. First, cells respond to an increment in extracellular cAMP concentration, adapt within minutes to the new cAMP concentration, and remain adapted as long as a constant level of cAMP persists. Serial increments in the level of extracellular cAMP evoke successive signaling responses followed by adaptation to each new stimulus concentration. The magnitude of each response depends on both the initial and final cAMP concentration. Second, when the cAMP stimulus is withdrawn, adaptation decays (deaptation) with a halftime of 3–4 min. Recovery of full sensitivity occurs 15 min after removal of the stimulus (4–6).

Earlier work suggested that adaptation is controlled by the level of receptor occupancy and is independent of the process that activates the adenylate cyclase (5). This conclusion could be tested directly by applying the cAMP stimulus in the presence of a reversible inhibitor of adenylate cyclase activa-

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tion and determining how adaptation is affected. If adaptation depends on activation of the enzyme or the resulting rise in intracellular cAMP, then, by inhibiting activation, adaptation would also be prevented. If this is the case, then the response should not be attenuated after pretreatment with the inhibitor plus cAMP. If, however, adaptation is independent of activation of the enzyme and is not directly inhibited by the drug, adaptation should proceed normally. In this case pretreatment with cAMP in the presence of inhibitor should result in an attenuation in the subsequent responsiveness that is the same as in cells pretreated with cAMP alone. We chose caffeine as the inhibitor since it rapidly blocks the activation of the adenylate cyclase. The effect is specific in that chemotaxis, light scattering, the rise in cGMP, binding of cAMP to surface receptors, phosphodiesterase, or adenylate cyclase activity in vitro are not inhibited (M. Brenner, personal communication). We show here that the effect of caffeine is rapidly reversible and we use it as a tool to demonstrate that adaptation is independent of activation of the adenylate cyclase.

MATERIALS AND METHODS

Growth and development of *D. discoideum* strain NC-4, [^{3}H]adenosine labeling, measurements of secretion rates, and purification of [^{3}H]cAMP have been previously described (7, 8). All the experiments reported here were carried out 5–9 h after harvesting cells from growth plates. Development buffer (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 1 mM $MgSO_4$, and 200 μM $CaCl_2$) was used for development and perfusion. Two four-place perfusion apparatuses were used so that eight identical filters of amebas were monitored in each experiment. Total radioactivity per filter was 2–6 × 10^6 cpm. cAMP (A9501) and caffeine (C-0750) were purchased from Sigma Chemical Co. (St. Louis, MO).

RESULTS

As previously demonstrated, when the stimulus concentration was held constant by rapid perfusion with 2 × 10^{-6} M cAMP, the [^{3}H]cAMP secretion rate rose and fell with a characteristic time course (Fig. 1*A*). The addition of 5 mM caffeine with the 2 × 10^{-6} M cAMP stimulus completely abolished the response (Fig. 1*B*). As shown in Fig. 1*C*, 50% inhibition occurred at 0.6 mM and complete inhibition at 2 mM caffeine. The same inhibition curve was observed at cAMP stimulus concentrations ranging between 10^{-9} and 10^{-4} M, indicating noncompetitive inhibition. For responses partially inhibited by caffeine, the rate of [^{3}H]cAMP secretion rose and fell with the same time course as untreated controls. Caffeine reduced the magnitude of the response but did not alter the kinetics. Since the degree of inhibition did not depend on the cAMP stimulus concentration, the collective data from many experiments are presented.

To investigate the reversibility of caffeine we tested cellular responsiveness to cAMP after pretreatment with caffeine. The response of control cells to a stimulus of 2 × 10^{-8} or 10^{-6} M cAMP is shown in Fig. 2, *A* and *B*, respectively. Pretreatment with caffeine for 8 min had only a slight inhibitory effect on the response when the perfusion buffer was switched from caffeine to the cAMP stimulus (Fig. 2, *C* and *D*). The average magnitude of the response after caffeine pretreatment was 84.4 ± 18% of the control (23 determinations). The slight inhibition did not depend on the duration of pretreatment (up to 22 min). Thus, it appears that caffeine rapidly blocks the signaling response and that its effect is quickly reversible.

As described above, the decline in the [^{3}H]cAMP secretion rate during persistent stimulation reflects an adaptation process that causes a decrease in adenylate cyclase activity in vivo.

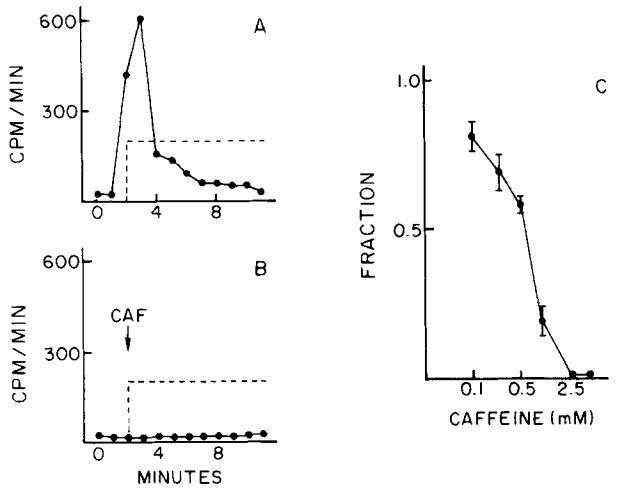

FIGURE 1 Inhibition of cAMP signaling response by caffeine. Rates of [^{3}H]cAMP secretion were measured as described. Application of cAMP stimuli is denoted by the dashed rectangles. The start of addition of caffeine is denoted by the arrow. (*A*), 2 × 10^{-6} M cAMP; (*B*), 2 × 10^{-6} M cAMP plus 5 mM caffeine; (*C*), the amount of [^{3}H]cAMP secreted in response to a stimulus of cAMP plus the indicated dose of caffeine normalized to the amount secreted in response to the same dose of cAMP in the absence of caffeine. Data from eight sets of experiments were pooled. In each set, the inhibition curve was performed at one stimulus concentration. The stimulus concentrations ranged from 10^{-8} to 10^{-4} M cAMP. Error bars indicate SE.

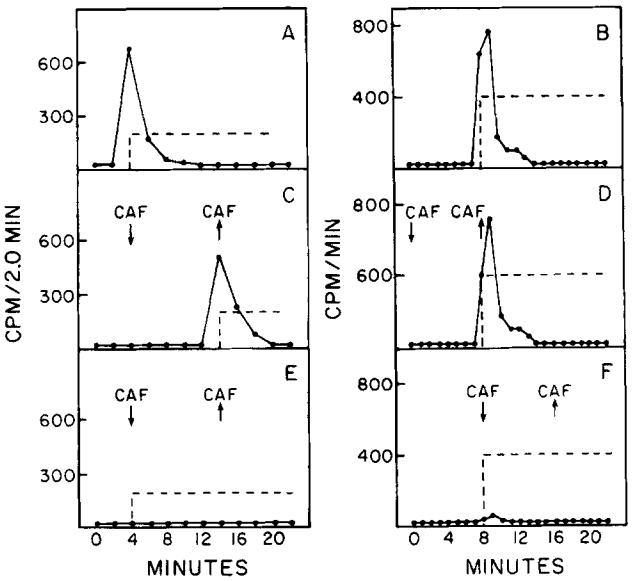

FIGURE 2 Reversibility of caffeine and adaptation to cAMP in the presence of caffeine. Addition and withdrawal of caffeine is denoted by the arrows. The stimulus in *A*, *C*, and *E* was 2 × 10^{-8} M cAMP (shown by the dashed rectangles) and the caffeine concentration was 3 mM. The stimulus in *B*, *D*, and *F* was 10^{-6} M cAMP (also shown by the dashed rectangles) and caffeine was 2 mM. (*A* and *B*) The response to the test stimulus; (*C* and *D*) the response to the test stimulus after pretreatment with caffeine for 8 min; (*E* and *F*), the response to the test stimulus after pretreatment with caffeine plus cAMP for 8 min.

Does adaptation depend on activation of the enzyme or the resulting rise in intracellular cAMP ? To answer these questions we applied cAMP plus caffeine for 8 min; we then withdrew caffeine and replaced it by the same concentration

of cAMP. As shown in Fig. 2, *E* and *F*, when the stimulus was switched from cAMP plus caffeine to cAMP the cells did not respond. Since it was shown above that the effects of caffeine are readily reversible (Fig. 2, *C* and *D*), the loss of responsiveness observed in Fig. 2, *E* and *F* must have been due to cAMP. This observation suggests that, even when the activation of the adenylate cyclase is prevented, cells adapt to the extracellular cAMP stimulus.

To substantiate this conclusion we carried out a more detailed analysis of the adaptation process under conditions where activation of the adenylate cyclase was blocked. Fig. 3 illustrates an experiment to assess the extent of adaptation by observing the response to sequential increments in the cAMP stimulus concentration. A stimulus of 10^{-8} M cAMP was applied for 8 min and then directly increased to 10^{-6} M cAMP (Fig. 3*A*). After response and adaptation to the initial stimulus increment (10^{-8} M cAMP), the further increment in the stimulus concentration (to 10^{-6} M) elicited a second response. The magnitude of the second response was attenuated due to adaptation to the initial stimulus. The sum of the magnitudes of the responses to the two increments equaled the magnitude of the response elicited when the highest stimulus was applied directly (Fig. 3*B*). Fig. 3*C* shows the same experiment as Fig. 3*A* except that caffeine was present during the initial stimulus and was removed when the cAMP stimulus was incremented. The initial response to 10^{-8} M cAMP was blocked by caffeine, but there was a response when the caffeine was removed and the cAMP concentration incremented to 10^{-6} M. If cells adapted in the presence of caffeine, the response to 10^{-6} M cAMP should have been attenuated to the same degree whether or not the initial 10^{-8} M-cAMP stimulus contained caffeine. Note that the responses were identical (compare second response in Fig. 3, *A* with that in Fig. 3, *C*). If adaptation had depended on activation of the adenylate cyclase the response in Fig. 3*C* should have been the same as that to 10^{-6} M cAMP (Fig. 3*B*). As shown previously when the stimulus was not incremented, there was no response upon caffeine removal (Fig. 3, *D*).

In Fig. 4, experiments similar to that shown in Fig. 3 are summarized. The first stimulus, ranging between 10^{-9} and 10^{-5} M cAMP, was applied and followed directly by a second stimulus of a saturating concentration of cAMP ($\geq 10^{-6}$ M). The integrated cellular response to the second cAMP stimulus, when the response to the first stimulus was blocked by caffeine, is shown (symbols). Note that the response to the second cAMP stimulus decreased as the cAMP concentration of the first stimulus increased. The magnitudes of these responses were identical to those observed when the initial response was not blocked by caffeine (solid line). These observations suggest that adaptation proceeds to the same extent whether or not activation of adenylate cyclase is blocked.

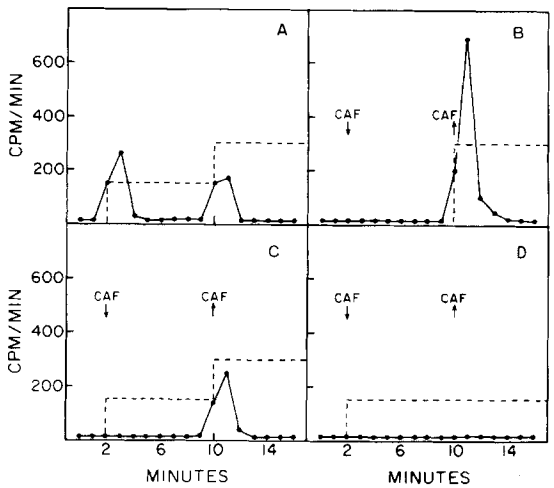

FIGURE 3 Response to stimulus increment following pretreatment with caffeine and cAMP. The cAMP stimuli (shown by the dashed rectangles) were as follows: (*A*) 10^{-8} M cAMP to 10^{-6} M cAMP and (*B*) 10^{-6} M cAMP; (*C*) 10^{-8} M cAMP to 10^{-6} M cAMP and (*D*) 10^{-8} M cAMP. 2 mM caffeine was applied and withdrawn as indicated by the arrows.

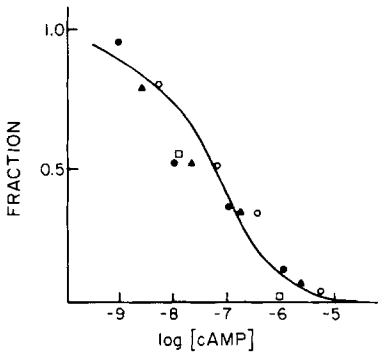

FIGURE 4 Response to a saturating stimulus following pretreatment with caffeine and increasing cAMP. The amount of [^{3}H]cAMP secreted in response to a saturating dose of cAMP ($\geq 10^{-6}$ M) after pretreatment for 7.5 min with 2 mM caffeine and the indicated dose of cAMP is shown by the symbols (from four independent experiments) as fraction of control. The control response was the response to the saturating dose ($\geq 10^{-6}$ M cAMP) after pretreatment for 7.5 min with 2 mM caffeine alone. The solid line shows the response to a saturating dose of cAMP ($\geq 10^{-6}$ M) after pretreatment with the indicated dose of cAMP for 7.5 min in the absence of caffeine (data pooled from three independent experiments).

Next, we determined whether the rate of adaptation depended on the activation of adenylate cyclase. Cells were treated with 10^{-6} M cAMP plus caffeine for short time periods, caffeine was withdrawn, and the same dose (10^{-6} M cAMP) was applied. Fig. 5*A* shows the control response and the responses following pretreatment with 10^{-6} M cAMP plus caffeine for 30 s, 1 or 2 min. Note that the cells did respond when the stimulus was switched to cAMP after short pretreatments with cAMP plus caffeine, but, as the duration of pretreatment increased, the magnitude of the response decreased. The magnitudes of the responses in this experiment and others are plotted in Fig. 5*B* as fractions of the control response to 10^{-6} M cAMP (open symbols). As the time of pretreatment with caffeine and cAMP increased from 0 to 8 min, the fractional size of the response to cAMP decreased from 1 to 0. The solid curve (closed symbols) shows the calculated rate of adaptation in control cells. This curve is that fraction of the control response that occurred after the indicated time. The similarity between the calculated curve and the experimental points indicates that the rate of adaptation is approximately the same in the presence and absence of adenylate cyclase activation.

Another characteristic of the response-adaptation system is the cell's capacity to recover (deadapt) once a stimulus has been removed. As shown in Fig. 6*A*, when cells were treated with 10^{-7} M cAMP, the stimulus was removed and then the same stimulus was reapplied, 15 min was sufficient time for cells to recover nearly complete responsiveness. To examine whether the adaptation that occurs in the absence of adenylate cyclase activation is reversible in the same way, the first response was inhibited by caffeine, cAMP and caffeine were

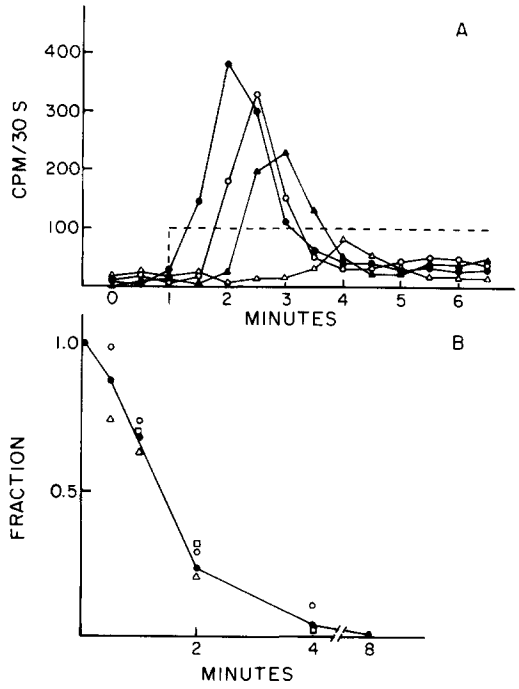

FIGURE 5 Kinetics of adaptation in the presence of caffeine. Cells were pretreated with a 10^{-6} M-cAMP stimulus plus 2 mM caffeine for short times and the stimulus was changed to 10^{-6} M cAMP alone (dashed rectangle shows total time of treatment with cAMP). In *A*: ●, no caffeine and cAMP pretreatment; O, switch from cAMP plus caffeine to cAMP after 30 s; ▲, after 1 min; Δ, after 2 min. In *B*: the open symbols (from three independent experiments) represent the amount of [^{3}H]cAMP secreted in response to a 10^{-6} M-cAMP stimulus after pretreatment with 2 mM caffeine plus 10^{-6} M cAMP for the indicated time, as compared with an unpretreated control. The solid line (closed symbols) was calculated from a control response and represents the fraction of the response that occurred after the time indicated.

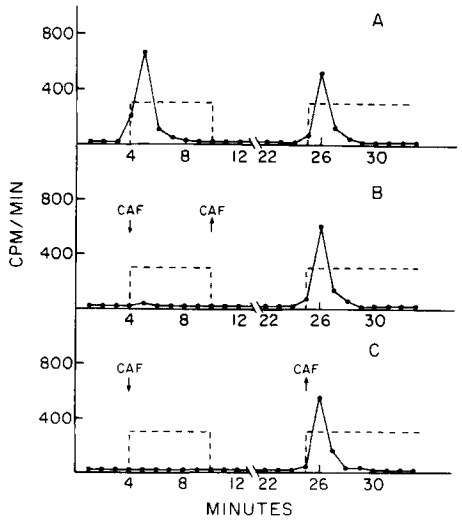

FIGURE 6 Deadaptation in the presence of caffeine. Two identical stimuli of 10^{-7} M cAMP (represented by dashed rectangles) were separated by a recovery interval of 15 min. 2 mM caffeine was applied and withdrawn as shown by the arrows. (*A*) control; (*B*) deadaptation in the absence of caffeine after adaptation in the presence of caffeine; (C) adaptation and deadaptation in the presence of caffeine.

removed, and after 15 min the cAMP stimulus was reapplied. When cAMP and caffeine were removed, these cells recovered complete responsiveness within the same time period as the control cells (Fig. 6*B*). In the experiment shown in Fig. 6*C*, caffeine was also included during the recovery interval. Again, the cells become fully responsive after 15 min. Thus, it appears that the adaptation that occurs in the absence of the response is reversible and that deadaptation is also not affected by caffeine.

DISCUSSION

In sensitive *D. discoideum* amebas an increase in the occupancy of surface cAMP receptors leads to activation of adenylate cyclase. When a constant level of stimulus is maintained, a gradual adjustment in cellular sensitivity occurs. Referred to as adaptation, this process causes the activity of the adenylate cyclase to return to the prestimulus level within a few minutes regardless of persistent cAMP stimulation. We have previously proposed that adaptation is a process controlled directly by surface receptor occupancy and is independent of activation of the adenylate cyclase (5). Experiments designed to test this hypothesis have yielded the following results. (*a*) When cells are pretreated with cAMP plus caffeine for 8–10 min and the stimulus is switched to the same concentration of cAMP, there is no response. (*b*) When cells are pretreated with cAMP plus caffeine for 8–10 min, the caffeine is withdrawn, and the cAMP stimulus is increased to a saturating dose, the observed responses are attenuated to the same extent as in controls pretreated with the same concentration of cAMP alone. (*c*) When cells are pretreated with cAMP plus caffeine for 0–8 min and the stimulus is switched to cAMP, the magnitude of the response decreases with the time of pretreatment. The kinetics of this decrease match those of adaptation in control cells. (*d*) When cells are pretreated with cAMP plus caffeine for 8 min and the cAMP stimulus is withdrawn, responsiveness is recovered within the same time period as control cells. These observations suggest that adaptation and deadaptation occur to the same extent and at the same rate whether or not there is concomitant activation of the adenylate cyclase. Adaptation thus depends closely on the fraction of occupied surface receptors but not on the activation of the enzyme or the ensuing rise in intracellular cAMP.

In vertebrate cells, hormones that activate adenylate cyclase also lead to specific desensitization of the activity. By using the adenylate cyclase deficient variant (cyc$^-$) of the S49 lymphoma cell line, it was shown that desensitization is independent of adenylate cyclase activation (9). In this respect, adaptation in *D. discoideum* is similar to desensitization in vertebrate cells, although little is known about the molecular events that lead to the turn off of adenylate cyclase in either system. If future research reveals that receptors and adenylate cyclase in *D. discoideum* are linked via a GTP-regulatory protein as in vertebrates, it may be likely that the mechanisms of adaptation are also similar in the two systems.

Caffeine prevents activation of the adenylate cyclase in intact cells, yet binding of [^{3}H]cAMP and basal adenylate cyclase activity are unaffected (M. Brenner, personal communication). A similar effect is observed in several other instances. In the presence of NaN_3 or elevated osmolarity (>100 mM) and in the mutants N7 and Agip53, addition of cAMP does not lead to activation of the adenylate cyclase, yet [^{3}H]cAMP binding and basal adenylate cyclase are appar-

ently normal (1, 10, 11). These observations suggest that intermediate steps are involved in the pathway linking receptors to the enzyme. Caffeine must inhibit at an intermediate step but after the point at which adaptation occurs since adaptation is unaffected by the drug. Consequently, caffeine provides a useful tool for further studies of the response system. A potentially interesting reaction triggered by extracellular cAMP can be quickly categorized as to its possible involvement in activation or adaptation of adenylate cyclase by its sensitivity to caffeine.

The adaptation process is of fundamental importance in *D. discoideum*. It controls the kinetics of cAMP secretion, enables delineation of the boundaries between adjacent aggregation territories, and is involved in the formation of centers within cell monolayers and the generation of spontaneous oscillations in cellular cAMP levels in cell suspensions. Several mathematical models describing the cAMP signaling response have been proposed. In the models, the response is described by a series of simultaneous differential equations. In any such model there must be a parameter that leads to attenuation of the response within a few minutes. So far, the parameters proposed have been depletion of the substrate (ATP) or cAMP-dependent phosphorylation of the adenylate cyclase, reactions that depend on the response (12, 13). However, all such "feedback" models are ruled out by the demonstration herein that adaptation occurs normally in the absence of the response. Other work indicates that there is no detectable depletion of the substrate (ATP), no effect of protein synthesis inhibitors, and no significant loss of surface cAMP binding sites during the response (14, 15). On the basis of these observations and the kinetics of adaptation and deadaptation, we speculate that the adaptation process may involve a reversible covalent modification of a component in the pathway linking receptors to adenylate cyclase.

We would like to thank Dr. M. Brenner for valuable discussion and Ms. S. Metzger for typing.

This research was supported by National Institutes of Health (NIH) grant GM 28007 to P. N. Devreotes.

A. Theibert is a predoctoral fellow supported by NIH Training grant GM07445. P. N. Devreotes is an American Cancer Society Junior Faculty Research Fellow.

Received for publication 20 December 1982, and in revised form 23 March 1983.

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