Cyclic 3',5'-AMP Relay in *Dictyostelium discoideum*

V. Adaptation of the cAMP Signaling Response

during cAMP Stimulation

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ABSTRACT   In *Dictyostelium discoideum*, extracellular cAMP activates adenylate cyclase, which leads to an increase in intracellular cAMP and the rate of cAMP secretion. The signaling response to a constant cAMP stimulus is terminated after several minutes by an adaptation mechanism. The time-course of adaptation to stimuli of 10^{-6} or 10^{-7} M cAMP was assessed. We used a perfusion technique to deliver defined cAMP stimuli to [^{3}H]adenosine-labeled amoebae and monitored their secretion of [^{3}H]cAMP. Amoebae were pretreated with 10^{-6} or 10^{-7} M cAMP for periods of 0.33–12 minutes, and then immediately given test stimuli of 10^{-8} M to 2.5×10^{-7} M cAMP. The response to a given test stimulus was progressively attenuated and finally extinguished as the duration of the pretreatment stimulus increased. During pretreatment with 10^{-6} M cAMP, the rates of attenuation could be ranked according to the concentration of the test stimulus. The responses to test stimuli of 10^{-8}, 5×10^{-8}, 10^{-7}, or 2.5×10^{-7} M cAMP were extinguished after ~1, 2.25, 2.5, and 10 min, respectively. 1.5 min of stimulation with 10^{-7} M cAMP was necessary to extinguish the response of a test stimulus of 10^{-8} M cAMP. Our data suggest that adaptation begins within 20 s of stimulation, rises rapidly for ~2.5 min, and reaches a plateau after ~10 min. The absolute rate of rise was faster during pretreatment with 10^{-6} than with 10^{-7} M cAMP. These results support a working hypothesis in which the occupancy of surface cAMP receptors leads to changes in two opposing cellular processes, excitation and adaptation, that control the activity of *D. discoideum* adenylate cyclase.

The aggregation of *Dictyostelium discoideum* is mediated by propagated signals of cyclic adenosine 3',5'-monophosphate (cAMP). The signaling response has been studied biochemically (5, 13, 14). The binding of cAMP to cell surface receptors (7, 8) leads to the activation of adenylate cyclase (5, 12), an increase in the level of intracellular cAMP, and secretion of the newly-synthesized cAMP (5). If the stimulus is withdrawn during the response, signaling promptly subsides. Signaling responses also terminate spontaneously after a few minutes of sustained stimulation as a result of an adaptation process (3). The mechanism of adaptation appears to involve a reversible, time-dependent decrease in sensitivity to cAMP stimuli. Serial increments in the level of extracellular cAMP evoke successive signaling responses followed by adaptation to each new stimulus concentration. During aggregation, adaptation presumably regulates the magnitude and duration of cAMP signaling

responses and prevents amoebae from responding perpetually to their own secretions.

We have previously shown that adaptation begins to increase before the signaling response terminates, persists after the decline of the response, and decays with first-order kinetics as soon as the cAMP stimulus is removed (3, 6). In this investigation, we sought to define the time-course for the rise in adaptation upon the introduction of a cAMP stimulus. As a working hypothesis, we assumed that a stimulus, perceived as an increase in the fractional occupancy of cell surface cAMP receptors, produces changes in both an excitation process that leads to activation of adenylate cyclase, and an opposing process, adaptation, that blocks such activation (6).

If this hypothesis is valid, the level of adaptation might be detected at any time during the signaling response by interrupting the stimulus and substituting a test stimulus of lower

magnitude. We reasoned that the first stimulus would not diminish the response to the test stimulus if the level of adaptation were not increased or if adaptation disappeared immediately upon reducing the stimulus concentration. However, if the level of adaptation had increased in a graded manner throughout the first stimulus and persisted during the second, the second stimulus would elicit responses whose magnitudes were diminished according to the duration and perhaps the concentration of the first stimulus. We therefore measured the effect of stimulation at a high concentration of cAMP on the signaling response to a juxtaposed stimulus of lower concentration.

MATERIALS AND METHODS

The NC-4 strain of *D. discoideum* was used in all experiments. Conditions for growth, [3H]adenosine labelling, development to the aggregation stage, and execution of experiments were as described in references 5 and 6. Amoebae were transferred to a four-filter perfusion apparatus when the first signs of aggregation were visible and synchronized by perfusion with buffer for 8–15 min, with 10^{-8} M cAMP for 3 min, and with buffer for an additional 15–18 min. A specific stimulus or sequence of stimuli was then administered. The [3H]cAMP secreted by amoebae was purified by chromatography on Bio-Rad AG 50W-X4 and neutral alumina columns (5).

Experimental Strategy

To detect changes in adaptation before the termination of the response, a cAMP stimulus was applied for a specified duration (pretreatment stimulus) and immediately followed by a second stimulus of lower concentration (test stimulus). We reasoned that the magnitude of the response to the test stimulus would be attenuated, compared to that elicited without pretreatment, according to the extent of adaptation evoked by the first cAMP stimulus. As a control, an identical filter of amoebae perfused in parallel was stimulated with the pretreatment stimulus alone, perfused with buffer for 15–25 min to allow deadaptation, and then given the test stimulus.[1] Responses were quantitated as the total [3H]cAMP (cpm) secreted during the stimulus or stimulus sequence, corrected for small differences in the numbers of cells per filter.

To evaluate the impact of pretreatment on the response to the test stimulus, we calculated an attenuation ratio = (response to $[C_1, t_1 \rightarrow C_2, t_2]$ − response to $[C_1, t_1 \rightarrow 0, t_2]$)/(response to $[C_2, t_2]$). $(C_1, t_1 \rightarrow C_2, t_2)$ denotes the sequence in which the pretreatment stimulus C_1 was applied for duration t_1, and then immediately followed by a lower test stimulus, C_2, of duration t_2. $(C_1, t_1 \rightarrow 0, t_2)$ represents the pretreatment stimulus C_1 when applied for duration t_1 and then removed.[2] (C_2, t_2) indicates the control stimulus at the lower concentration, C_2, for duration t_2, given without an immediately preceding pretreatment stimulus.[1] The attenuation ratio = 1 if the pretreatment had no effect on the response to the

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[1] We have observed that cAMP stimuli lead to an irreversible reduction in the size of responses to subsequent stimuli (6). This inhibition of responsiveness may be unrelated to the adaptation mechanism. To exclude this effect from the assessment of adaptation produced by the pretreatment stimulus, the control response to the second stimulus was routinely measured 15–25 min after stimulation with the pretreatment stimulus and used for the denominator in the attenuation ratio. In most experiments, another filter of amoebae was stimulated with the second stimulus without prior stimulation at the higher cAMP concentration to assess the degree of irreversible reduction in responsiveness caused by the larger stimulus. The response elicited by the second stimulus in the absence of pretreatment was not used to calculate the attenuation ratio, but is listed in the figure legends.

[2] Even though identical filters of amoebae were stimulated in parallel, the magnitude of the response to the first stimulus before the removal or lowering of external cAMP typically varied by 5–20% (and by as much as 50% in three cases). The amount of [3H]cAMP (cpm) in the fractions collected during the application of the first stimulus, C_1, plus the amount in the next fraction, was determined for each of the two filters. (The following fraction was used as the cut-off, because it was identical in cases where the total responses elicited from cells on the two filters were identical.) Before calculation of the attenuation ratio, the magnitude of the response to the first stimulus given alone, $(C_1, t_1 \rightarrow 0, t_2)$, was corrected for this difference between filters.

test stimulus. If the response to the test stimulus was abolished by pretreatment, the ratio = 0.

RESULTS

Effect of 10^{-6} M cAMP Pretreatment on Responses to Test Stimuli of 10^{-8} M cAMP

The time-course of [3H]cAMP secretion by prelabeled amoebae during a 5-min stimulus of 10^{-8} M cAMP is shown in Fig. 1 a. The rate of secretion of [3H]cAMP rose to a peak after 2.5 min and then abruptly declined. The time-course of a response to a 5-min stimulus of 10^{-6} M cAMP was similar, but the magnitude of the changes in the rate of cAMP secretion were typically fivefold larger. When 10^{-6} M cAMP was removed after 20 s, the response was drastically curtailed (Fig. 1 b).
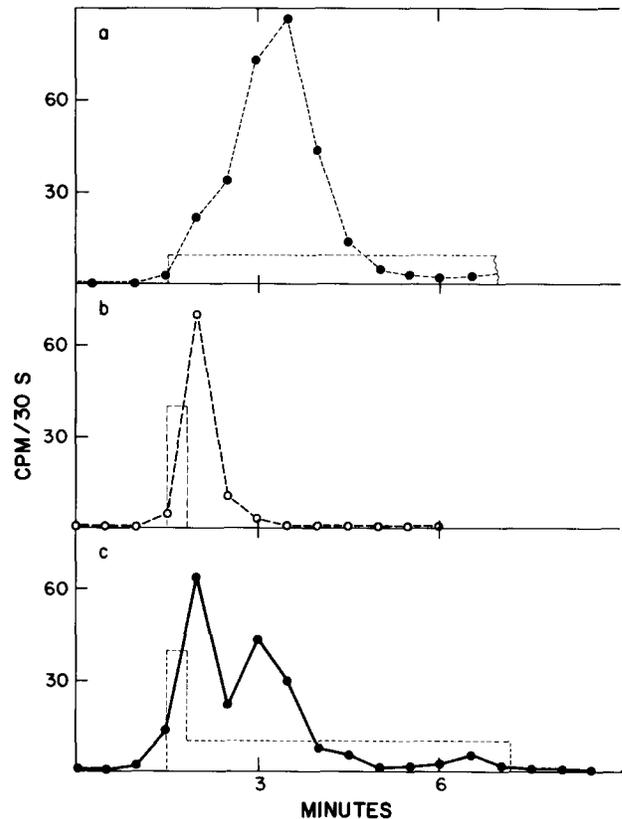

FIGURE 1   Effect of a 20-s pretreatment with 10^{-6} M cAMP on the response to 10^{-8} M cAMP. [3H]adenosine-labeled amoebae at the early aggregation stage were transferred to filters and synchronized before beginning the experiment. Application of cAMP stimuli is denoted by the dashed rectangles. Fractions were collected every 0.5 minutes for analysis of [3H]cAMP. Total radioactivity associated with each filter was 3.8 × 10^6 cpm. (*a*) (--●--) Response to a 5.5-min (t_2) stimulus of 10^{-8} M cAMP (C_2) (10^{-8} M cAMP, 5.5 min). (*b*) (——○——) Response to a 20-s (t_1) stimulus of 10^{-6} M cAMP (C_1) (10^{-6} M cAMP, 20 s → 0). The radioactivity in each fraction has been multiplied by 1.4 to adjust for differences between filters of amoebae.[2] (*c*) (——●——) Response to a 20-s (t_1) stimulus of 10^{-6} M cAMP (C_1) followed immediately by a 5.5-min (t_2) stimulus of 10^{-8} M cAMP (C_2) (10^{-6} M cAMP, 20 s → 10^{-8} M cAMP, 5.5 min). The attenuation ratio was 0.47, but without adjustment of the response in *b*, the ratio equaled 0.40. The 10^{-8} M cAMP stimulus in *a* was separated from a preceding 20-s 10^{-6} M stimulus by 15 min of perfusion with M-KK₂ (5). The magnitude of the illustrated response to the 10^{-8} M stimulus was 82% of that elicited by a 5.5-min 10^{-8} M cAMP stimulus administered to another filter of amoebae not previously stimulated with 10^{-6} M cAMP.

When a 20-s, 10^{-6} M cAMP stimulus was followed by 10^{-8} M cAMP, the rate of secretion rapidly declined, but then rose to a second peak 2 min from the beginning of the first stimulus (Fig. 1 c). The time-course of cAMP secretion thus had a notched appearance. It appeared that the magnitude of the response to 10^{-8} M cAMP was reduced by pretreatment at 10^{-6} M cAMP, presumably as a result of the accrual of adaptation during the first stimulus. The attenuation ratio, a measure of the effect of pretreatment on the response to the test stimulus, was 0.47. Surprisingly, the magnitude of the entire response to the compound stimulus was actually smaller than that to 10^{-8} M cAMP alone.

When the duration of the 10^{-6} M cAMP stimulus was extended to 45 and 90 s, the magnitude of the resulting signaling response increased (Fig. 2 a and b; open circles). However, juxtaposed test stimuli of 10^{-8} M cAMP elicited
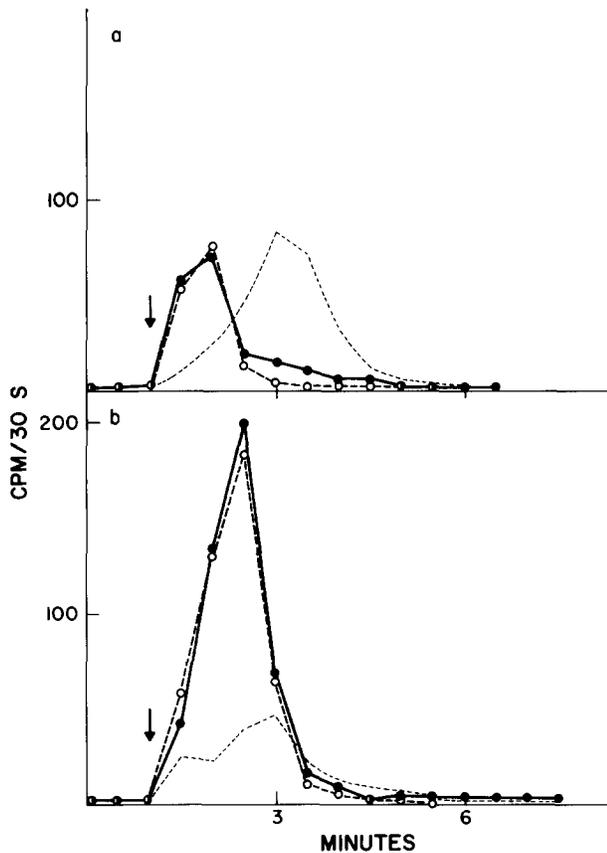

FIGURE 2   Effect of pretreatment with 10^{-6} M cAMP on responses to 10^{-8} M cAMP. Panels a and b each show results from a single experiment, carried out as described in Fig. 1. The onset of each cAMP stimulus or stimulus sequence is denoted by the arrow. (a) ●, The response to (10^{-6} M cAMP, 0.75 min → 10^{-8} M cAMP, 6 min). O, The response elicited from an identical filter of amoebae by (10^{-6} M cAMP, 0.75 min → 0). This filter was stimulated 20 min later with (10^{-8} M cAMP, 6 min) (----); the magnitude of the response was 68% of that elicited by an identical stimulus given to a third filter of amoebae without prior 10^{-6} M cAMP exposure. The attenuation ratio was 0.12. (b) ●, The response to (10^{-6} M cAMP, 1.5 min → 10^{-8} M cAMP, 5.5 min). O, The response to (10^{-6} M cAMP, 1.5 min → 0); this stimulus was followed 20 min later by (10^{-8} M cAMP, 7 min) (----). The response to the latter stimulus was 52% of that elicited by the same stimulus given to another filter of amoebae without prior 10^{-6} M cAMP stimulation. The attenuation ratio was 0.12. Total radioactivity associated with each filter was 2.1 × 10^6 cpm (a) and 2.9 × 10^6 cpm (b).
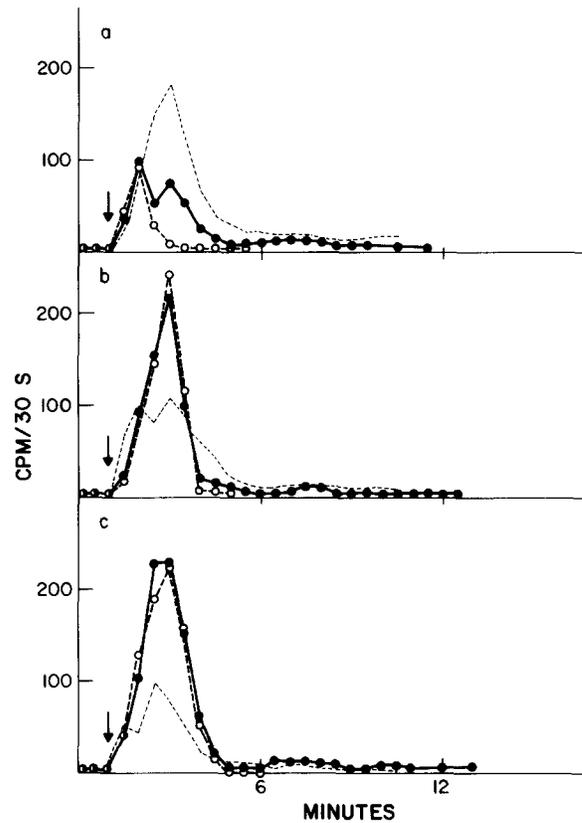
FIGURE 3   Effect of pretreatment with 10^{-6} M cAMP on responses to 5 × 10^{-8} M cAMP. Panels a, b, and c each show results from a single experiment, carried out as described in Fig. 1. The onset of each cAMP stimulus of stimulus sequence is shown by the arrow. (a) ●, The response to (10^{-6} M cAMP, 1 min → 5 × 10^{-8} M cAMP, 10 min). O, The response to (10^{-6} M cAMP, 1 min → 0); the radioactivity in fraction has been multiplied by 0.77 to adjust for differences between filters.[2] This filter of amoebae was also stimulated 20 min later with (5 × 10^{-8} M cAMP, 10 min) (----); the magnitude of this response was identical to that elicited by 5 × 10^{-8} M cAMP applied to a third filter of amoebae without a prior 10^{-6} M stimulus. The attenuation ratio was 0.38; without adjustment of the response to (10^{-6} M cAMP, 1 min → 0); it was equal to 0.32. (b) ●, The response to (10^{-6} M cAMP, 2 min → 5 × 10^{-8} M cAMP, 10 min). O, The response to (10^{-6} M cAMP, 2 min → 0); each fraction has been multiplied by 0.90 to adjust for differences between filters.[2] The 2-min stimulus was followed 20 min later by (5 × 10^{-8} M cAMP, 10 min) (----); the response to this test stimulus was 78% of that elicited from a third filter of amoebae stimulated with 5 × 10^{-8} M cAMP alone. The attenuation ratio was 0.10; if the response to (10^{-6} M cAMP, 2 min → 0) was not adjusted, this ratio equaled −0.01. (c) ●, The response to (10^{-6} M cAMP, 2.5 min → 5 × 10^{-8} M cAMP, 10 min). O, The response to (10^{-6} M cAMP, 2.5 min → 0); this stimulus was followed 20 min later by (5 × 10^{-8} M cAMP, 10 min) (---). The latter response was 73% of that elicited by the same stimulus given to a third filter of amoebae without prior 10^{-6} M cAMP stimulation. The attenuation ratio was 0.21. Total radioactivity associated with each filter was 6.0 × 10^6 cpm (a), 4.4 × 10^6 cpm (b), and 2.9 × 10^6 cpm (c).

progressively smaller responses (Figure 2 a and b; closed circles). Ultimately, the test stimulus elicited only very low rates of cAMP secretion so that the profile of cAMP secretion during the combined first and second stimuli resembled the response to the pretreatment stimulus given alone. The attenuation ratios approached 0 (ratios, 0.075–0.20) and changed very little when the duration of the 10^{-6} M stimulus was extended beyond 50–60 s.

Effect of 10^{-6} M cAMP Pretreatment on the Responses to Higher cAMP Test Stimuli

Although 45 s of 10^{-6} M cAMP pretreatment almost completely extinguished the response to a 10^{-8} M cAMP stimulus (Fig. 2a), a 5×10^{-8} M cAMP test stimulus still elicited considerable cAMP secretion when immediately preceded by a 1-min 10^{-6} M cAMP stimulus (Fig. 3a). However, the magnitude of the response to the 5×10^{-8} M test stimulus was only 38% of that elicited by a control 5×10^{-8} M cAMP stimulus. When the 10^{-6} M pretreatment was extended to 2–2.5 min, the release of cAMP during a juxtaposed stimulus of 5×10^{-8} M cAMP was greatly diminished (Fig. 3b and c). Thus, longer exposures to 10^{-6} M cAMP were necessary to attenuate responsiveness to 5×10^{-8} M cAMP as compared with 10^{-8} M cAMP test stimuli.

The response to a 10^{-7} M cAMP test stimulus after pretreat-
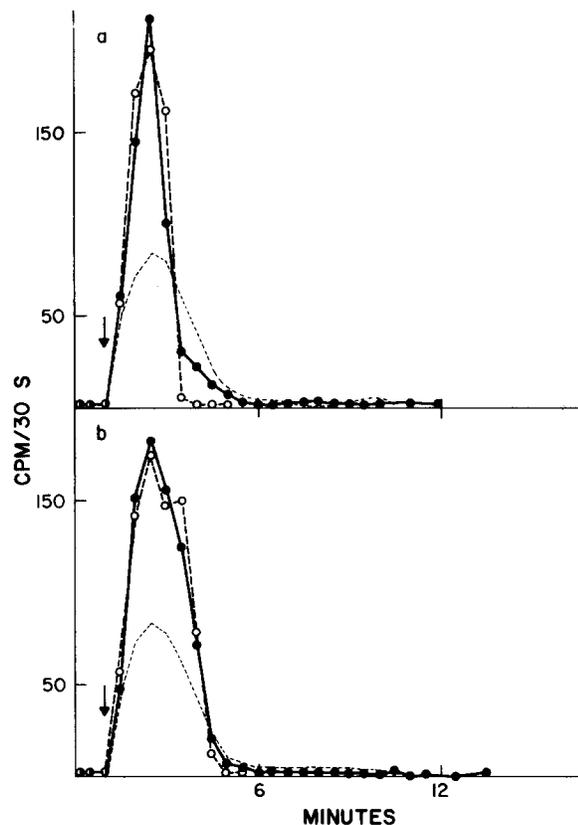

FIGURE 4 Effect of pretreatment with 10^{-6} M cAMP on responses to 10^{-7} M cAMP. Panels a and b show results from a single experiment, carried out as described in Fig. 1. Total radioactivity associated with each filter was 2.0×10^6 cpm. The onset of each cAMP stimulus of stimulus sequence is denoted by the arrow. (a) ●, The response to (10^{-6} M cAMP, 1.5 min → 10^{-7} M cAMP, 10 min). ○, The response to (10^{-6} M cAMP, 1.5 min → 0); the radioactivity in each fraction has been multiplied by 1.07.[2] The response to (10^{-7} M cAMP, 10 min), elicited 20 min after (10^{-6} M cAMP, 1.5 min → 0), is shown (- - - -). The attenuation ratio was 0.36, or 0.44 without adjustment of the response to (10^{-6} M cAMP, 1.5 min → 0). (b) ●, The response to (10^{-6} M cAMP, 2.75 min → 10^{-7} M cAMP, 10 min). ○, The response to (10^{-6} M cAMP, 2.75 min → 0); the radioactivity in each fraction has been multiplied by 0.95.[2] The response to (10^{-7} M cAMP, 10 min) was elicited 20 min after (10^{-6} M cAMP, 2.75 min → 0), and is shown (- - - -). The attenuation ratio was 0.33, but if the response to (10^{-6} M cAMP, 2.75 min → 0) was not adjusted, the ratio equaled −0.06.

ment with 10^{-6} M cAMP was affected in a manner similar to 5×10^{-8} M cAMP test stimuli. The secretion of cAMP was clearly prolonged when a 1.5-min, 10^{-6} M cAMP stimulus was lowered to 10^{-7} M cAMP as compared with the complete removal of cAMP (Fig. 4a). However, 2.75 min of stimulation at 10^{-6} M cAMP nearly abolished the response to a 10^{-7} M cAMP test stimulus (Fig. 4b).

Pretreatment of amoebae with 10^{-6} M cAMP for 1.25–6 min progressively attenuated their response to test stimuli of 2.5×10^{-7} M cAMP (Fig. 5). However, the attenuation was not nearly as marked when compared with test stimuli of lower concentrations. For example, a 1.25-min, 10^{-6} M cAMP stimulus reduced the size of the response to a juxtaposed 2.5×10^{-7} M stimulus to only 75% of a control (Fig. 5a). After 2 min of 10^{-6} M cAMP, which nearly extinguished the response to a 5×10^{-8} M cAMP test stimulus (Fig. 3b), the response to a 2.5×10^{-7} M cAMP test stimulus was still 56% of the control (Fig. 5b). Although the time-course of the response to this combined stimulus was similar to that elicited by a sustained 10^{-6} M cAMP stimulus (also shown in Fig. 5b), comparison of these two responses clearly shows that amoebae rapidly adjusted the size of their response when the external cAMP concentration was lowered. After 6 min of prior stimulation with 10^{-6} M cAMP, the response to a test stimulus of 2.5×10^{-7} M cAMP had declined to 20% of the control (Fig. 5c). When the 10^{-6} M stimulus was extended to 12 min, an attenuation ratio of 0.08 was obtained for a test stimulus of 2.5×10^{-7} M cAMP (not shown).

Effect of 10^{-7} M cAMP Pretreatment on Responses to Test Stimuli of 10^{-8} M cAMP

After 1 min of stimulation with 10^{-7} M cAMP, a test stimulus of 10^{-8} M cAMP still elicited cAMP secretion, although the amount was reduced as compared with responses to 10^{-8} M controls (attenuation ratios = 0.19, 0.31, 0.49 in three separate experiments). The response to a 10^{-8} M test stimulus dropped to 10% of controls after 1.5 min of a 10^{-7} M stimulus in three separate experiments. In contrast, stimulation with 10^{-6} M cAMP for only 45 s achieved the same degree of attenuation of responses to 10^{-8} M cAMP test stimuli. These data suggest that the rate of increase in the level of adaptation varies with the stimulus concentration.

DISCUSSION

Brief pretreatments with large cAMP stimuli attenuate and even extinguish the responses to smaller test stimuli applied immediately thereafter. Fig. 6, which summarizes our results, demonstrates several central features of the attenuation phenomenon. For any given test concentration, attenuation increased with the duration of pretreatment. The attenuation at any duration could be ranked according to the concentration of the test stimulus, with small stimuli more profoundly affected than larger test stimuli. Finally, attenuation of the responses to a test stimulus after a given duration of pretreatment increased with the concentration of the pretreating stimulus (Fig. 6, inset). These data suggest that pretreatment diminished cellular sensitivity in proportion to the level and duration of receptor occupancy rather than in an all-or-none or absolute fashion. Changes in responsiveness are not directly correlated with the amount of cAMP secreted during the pretreatment stimulus, because responses to the juxtaposed pretreatment and test
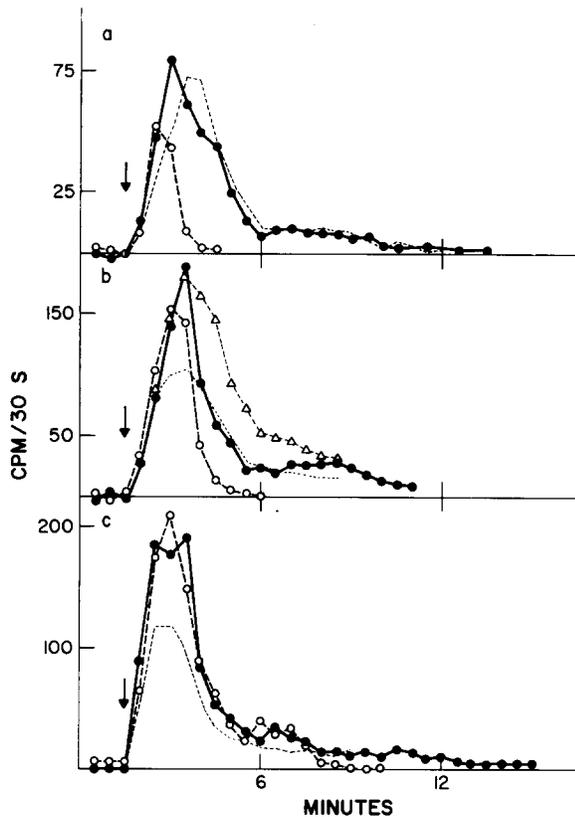
FIGURE 5 Effect of pretreatment with 10^{-6} M cAMP on responses to 2.5×10^{-7} M cAMP. Panels a, b, and c each show results from a single experiment carried out as described in Fig. 1. The onset of each stimulus or stimulus series is denoted by the arrow. (a) ●, The response to (10^{-6} M cAMP, 1.25 min → 2.5 × 10^{-7} M cAMP, 11 min). ○, The response to (10^{-6} M cAMP, 1.25 min → 0) and the radioactivity in each fraction has been multiplied by 1.11.[2] The response to (2.5 × 10^{-7} M cAMP, 11 min), elicited 20 min after (10^{-6} M cAMP, 1.25 min → 0), is shown (----). The attenuation ratio was 0.74; if the response to (10^{-6} M cAMP, 1.25 min → 0) was not adjusted, this ratio equaled 0.77. (b) ●, The response to (10^{-6} M cAMP, 2 min → 2.5 × 10^{-7} M cAMP, 8 min). ○, The response to (10^{-6} M cAMP, 2 min → 0); the radioactivity in each fraction was multiplied by 0.77.[2] This stimulus was followed 20 min later by (2.5 × 10^{-7} M cAMP, 8 min); the response is shown (----). The size of the response to the latter was 53% of that elicited from another filter of amoebae without prior stimulation at 10^{-6} M cAMP. The attenuation ratio was 0.56, or 0.42 if the response to (10^{-6} M cAMP, 2 min → 0) was not adjusted. Also illustrated in this panel is the response elicited by a 10^{-6} M cAMP stimulus of 8 min duration applied to a fourth filter of amoebae (△). This response was 1.25-fold larger than that to a stimulus of 2.5 × 10^{-7} M cAMP (given without any 10^{-6} M cAMP pretreatment). (c) ●, The response to (10^{-6} M cAMP, 6 min → 2.5 × 10^{-7} M cAMP, 8 min). ○, The response to (10^{-6} M cAMP, 6 min → 0), with the radioactivity in each fraction multiplied by 0.85.[2] The response to (2.5 × 10^{-7} M cAMP, 8 min), elicited 25 min after (10^{-6} M cAMP, 6 min → 0), is shown (----). The attenuation ratio was 0.20, but if the response to (10^{-6} M cAMP, 6 min → 0) was not adjusted, the ratio equaled −0.05. Total radioactivity associated with each filter was 1.1 × 10^6 cpm (a), 2.7 × 10^6 cpm (b), and 2.3 × 10^6 cpm (c).

stimuli were often smaller than to the test stimulus alone (e.g., Figs. 1 and 3 a, and reference 4).

Our results are compatible with a hypothesis in which the signaling response is regulated by the relative levels of excitation and adaptation processes (3, 6), as diagrammed in Fig. 7. We attribute the progressive diminution in cellular responsive-

ness, as the duration of pretreatment was extended, to a rise in the level of adaptation during pretreatment. If the test stimulus is given alone, adaptation does not null excitation at the cAMP receptor occupancy-specified level until after many minutes of stimulation (Fig. 7a). The effect of cAMP pretreatment on the response to a test stimulus is determined by (a) the level of preexistent adaptation produced by the pretreatment stimulus, dependent on its duration and concentration, and (b) the final excitation level produced by the test stimulus, proportional to the fractional occupancy of cAMP binding sites at the test concentration. If the preexistent adaptation level is still less than the new occupancy-specified level of excitation, a response continues until excitation and adaptation match at the new value (Fig. 7b). The magnitude of the response to the test stimulus is attenuated, because adaptation accrued during pretreatment. However, if the preexisting level of adaptation is already equal or greater to the maximal excitation level sustained by the test stimulus, little or no additional cAMP secretion is elicited (Fig. 7c and d). Under these conditions, the level of excitation must fall very rapidly to the new occupancy-specified level when the extracellular cAMP is lowered, because the signaling response stops as abruptly as when cAMP is completely removed (Figs. 2, 3b, and 4b).

Our working hypothesis further predicts that the response elicited by a juxtaposed pretreatment stimulus and smaller test stimulus should depend on the adaptation level already present at the onset of this sequence. This is demonstrated in the experiment illustrated in Fig. 8, in which a 1-min stimulus of 10^{-6} M cAMP was followed by 5 × 10^{-8} M cAMP. Amoebae responded to this test stimulus after 10^{-6} M pretreatment alone (Fig. 8a). However, this was not the case if amoebae were first stimulated with 5 × 10^{-8} M cAMP for 10 min; by the end of this stimulus, the rate of cAMP secretion was very low (i.e., almost full adaptation to 5 × 10^{-8} M cAMP had occurred). The subsequent increment to 10^{-6} M cAMP elicited a smaller
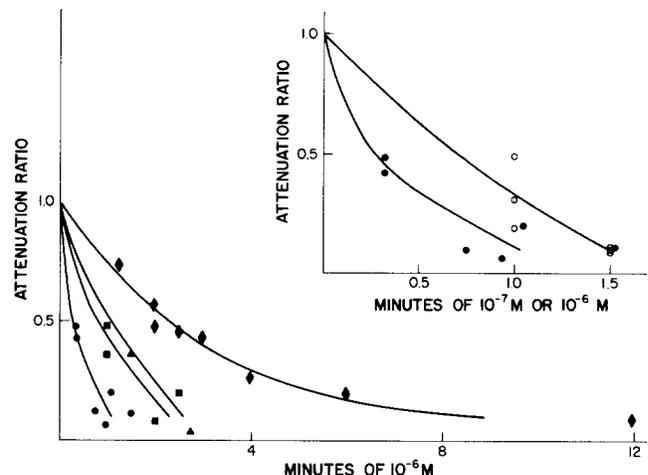

FIGURE 6 Dependence of attenuation ratio on the duration of pretreatment. The attenuation ratios obtained for test stimuli of 10^{-8} M cAMP (●), 5 × 10^{-8} M cAMP (■), 10^{-7} M cAMP (▲), and 2.5 × 10^{-7} M cAMP (◆) are plotted as a function of the duration of pretreatment with 10^{-6} M cAMP. The data were obtained from 12 experiments, each carried out on a different day, including those presented in Figs. 1–5. Inset: ○, Attenuation ratios obtained for 10^{-8} M cAMP test stimuli plotted as a function of the duration of pretreatment with 10^{-7} M cAMP. ●, The ratios for 10^{-6} M cAMP pretreatment of 10^{-8} M cAMP test stimuli, replotted for comparison. The former were obtained in three separate experiments, the latter in four separate experiments. All curves were drawn arbitrarily.
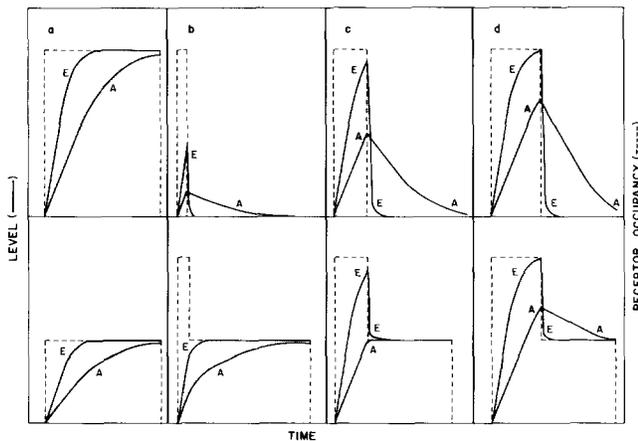
FIGURE 7 Application of excitation-adaptation scheme to cAMP signaling responses elicited by a juxtaposed sequence of high and low stimulus concentrations. This illustration is a qualitative diagram of hypothetical changes in the levels of the excitation (E) and adaptation (A) processes with different patterns of stimulation (see also reference 6). Dashed bars represent cAMP receptor occupancy. Shading denotes the signaling response when the level of E exceeds the level of A. (a) Control responses to high (upper panel) and low (lower panel) cAMP stimulus concentrations. E and A rise to the occupancy-specified level when receptor occupancy is held constant. (b–d) The remaining panels illustrate the changes in E and A when a cAMP stimulus of the high concentration is either removed or reduced to the lower concentration at various times. Upper panels: When cAMP is simply withdrawn, E and A return to their basal levels; the decline in E is more rapid than that in A, and no further responses are observed. Lower panels: (b) When receptor occupancy is lowered before E and A have reached the level specified by the lower receptor occupancy, E and A continue to rise, albeit at a slower rate, to the new level. The response to the smaller stimulus is diminished because the level of adaptation increased during the prior stimulus. (c) When receptor occupancy is lowered just when the level of A attained equals that specified by the new receptor occupancy, A no longer changes. The level of E falls rapidly to match A at the new occupancy-specified value; thus, the response rapidly subsides, just as if cAMP had been completely removed (upper panel). (d) The first stimulus is maintained until the levels of E and A surpass the value specified by the lower receptor occupancy. When occupancy is then reduced, E (rapidly) and A (slowly) fall to the lower value, promptly ending the response.

increase in cAMP secretion, and amoebae ceased responding when the stimulus was then returned to 5×10^{-8} M cAMP (Fig. 8b). This experiment provides evidence that the level of adaptation revealed by the test stimulus reflects not just the adaptation to the prior stimulus but the entire recent history of cellular adjustments in excitation and adaptation.

The level of adaptation appears to increase within 20 s of the onset of a stimulus (Fig. 1). We have estimated the time-course of the rise in adaptation during pretreatment with 10^{-6} M cAMP in terms of the duration of pretreatment at which cells became insensitive to the test stimuli of increasing concentration.[3] When the duration of pretreatment is just sufficient to extinguish the response to a lower test stimulus, the level of adaptation produced by pretreatment should correspond to the

[3] The values of attenuation ratios obtained for a given test stimulus approached zero with longer pretreatments of 10^{-6} M cAMP. This reflected very low rates of cAMP secretion during the test stimulus (cf. Figs. 2, 3b, 4b), which were similar to those observed when amoebae have almost fully adapted to a single stimulus after ~10 min of stimulation. Therefore, in this analysis we used the duration of 10^{-6} M cAMP pretreatment at which the attenuation ratio reached 0.10.

maximal level approached during a single sustained stimulus at the test concentration. These relationships were used to plot the relative level of adaptation as a function of the duration of a 10^{-6} M cAMP stimulus (Fig. 9). Adaptation rises from the onset of stimulation, increasing continuously to approach the level which extinguishes the response to 10^{-6} M cAMP itself. This analysis suggests that the adaptation process shapes the signaling response at all times rather than rising to terminate cAMP production after a period of several minutes.

Signaling responses to different stimulus increments peak and terminate at about the same time, suggesting that the relative rates of increase in excitation and adaptation levels are similar, regardless of the final receptor occupancy-specified level achieved. That a brief pretreatment of 10^{-6} or 10^{-7} M cAMP extinguished the response to a lower cAMP concentration implies that the absolute level of adaptation rises more rapidly during stimulation at higher concentrations of cAMP. Furthermore, the attenuation of responsiveness to 10^{-8} M cAMP was more rapid during pretreatment with a large increment, 10^{-6} M cAMP, than with a smaller increment, 10^{-7} M cAMP (Fig. 6, *inset*). Our results suggest that the rise in
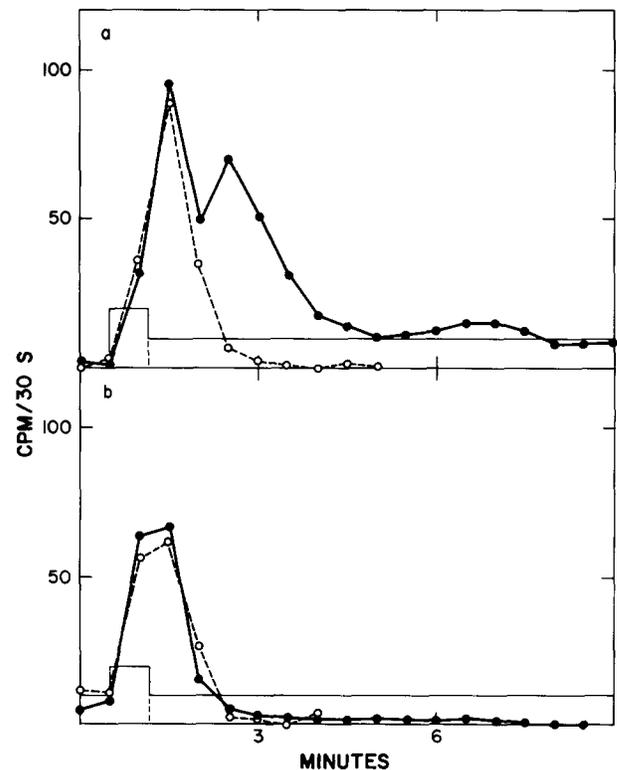

FIGURE 8 Effect of a preceding 5×10^{-8} M stimulus on the response to a sequence of 10^{-6} M and 5×10^{-8} M cAMP stimuli. Panels a and b show responses elicited in a single experiment carried out as in Fig. 1; panel a is taken from Fig. 3 a. The application and removal of cAMP stimuli are shown by the solid and dashed bars. Total radioactivity associated with each filter was 6×10^6 cpm. (a) The responses to a 1-min 10^{-6} M cAMP stimulus (O, removal of stimulus indicated by dashed vertical bar) and to a 1-min 10^{-6} M stimulus that was lowered to 5×10^{-8} M cAMP (●) are shown. (b) Two other filters of amoebae were first stimulated with 5×10^{-8} M cAMP for 10 min; the magnitude of these responses were identical. The final 30-s fraction, collected at the end of this stimulus, is shown. The stimulus concentration was then increased to 10^{-6} M cAMP for 1 min (as shown by the stimulus bars), and then either removed (O, removal indicated by dashed vertical bar) or reduced to 5×10^{-8} M cAMP (●).
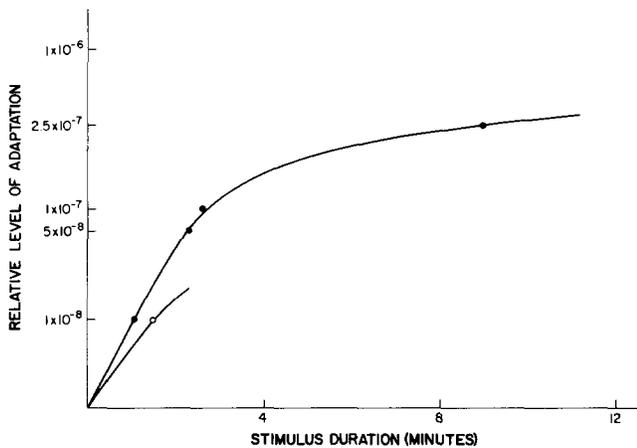
FIGURE 9 The increase in the relative level of adaptation during cAMP stimuli. The duration of pretreatment that reduced the attenuation ratio for a given test stimulus concentration to 0.10 was estimated from Fig. 6. The test stimulus concentration is plotted as a function of this duration, for pretreatment with 10^{-7} M cAMP (O) and 10^{-6} M cAMP (●). The scale of the ordinate axis is based on the relationship between the concentration of a cAMP stimulus and the relative magnitude of the response elicited (total cAMP secreted from stimulus onset to the spontaneous termination of the response by adaptation), under conditions where amoebae are not previously stimulated by exogenous cAMP (except for synchronization after loading in the perfusion systems). This information was obtained from these and other experiments.

adaptation elicited by 10^{-7} M cAMP reaches the 10^{-8} M cAMP occupancy-specified level at 1.5 min (Fig. 8, open circle); this level is achieved after only 1 min at 10^{-6} M cAMP. Although not shown, adaptation would presumably continue to rise to approach the level specified by 10^{-7} M cAMP at ~10 min, because at this time the signaling response to a sustained 10^{-7} M stimulus nearly subsides. Thus, both the final level and the absolute rate of increase in the adaptation process depend on the size of the increment in receptor occupancy.

Only two components involved in the cAMP signaling response have been clearly identified: surface cAMP receptors and adenylate cyclase. There are ~200,000 cAMP binding sites, of various affinities, per cell ($K_d = 2 \times 10^{-9}$ M $- 10^{-5}$ M) (7, 8). The adenylate cyclase is not exposed to the extracellular space in intact cells (reference 10 and unpublished observations), and its activation by external cAMP (5, 12) presumably occurs via these binding sites. However, it is evident that intermediate events link the occupancy of surface receptors to the activation of adenylate cyclase. Whereas the attainment of equilibrium binding of cAMP to surface sites is rapid ($t_{1/2}$ of a few seconds) (9), adenylate cyclase activity peaks only after 1–2 min (5). NaN$_3$, an inhibitor of the signaling response, blocks adenylate cyclase activation without interfering with the binding of cAMP to surface receptors or the activity of the preactivated enzyme in cell homogenates (5). Finally, when cAMP receptor occupancy is increased and maintained at a constant level, the activation of adenylate cyclase is transient (5). We have postulated that *D. discoideum* adenylate cyclase activity is controlled indirectly by cAMP receptor occupancy via two antagonistic cellular processes, excitation and adaptation (3, 6).

Although our results were obtained by use of a physiological approach, it is possible to speculate on the molecular basis of the excitation and adaptation processes. The adaptation process begins immediately at the onset of a stimulus, rising at an absolute rate proportional to the increment in receptor occu-

pancy and reaching the occupancy-specified level after ~10 min (see reference 6). Adaptation is maintained at this level until the stimulus is removed, and then spontaneously decays with first-order kinetics (6). The $t_{1/2}$ for both the rise and fall of the level of adaptation are ~3 min. This appears to hold for the increase in the level of adaptation elicited by any increment in stimulus concentration and for the decay in adaptation from any level (6). These properties suggest there is an adaptation effector that is turning over; its rate of formation depends instantly on receptor occupancy and its rate of destruction is first-order. Any change in receptor occupancy alters the steady-state level of this effector, but the $t_{1/2}$ of approach to the new steady-state level is always ~3 min, because this depends only on the rate constant of degradation. For example, if the adaptation effector was a covalently modified protein, the rate of modification would be controlled by receptor occupancy, and the rate of the reverse reaction would be first-order.

It is unlikely that adaptation involves a mechanism whereby functional binding sites are inactivated by their interaction with cAMP. We have pointed out that if there were a single class of binding sites, adaptation to even a low cAMP concentration would require inactivation of all sites (3). This is incompatible, however, with the observation that further signaling responses can be elicited by increasing the stimulus (3). One might argue that if multiple classes of binding sites existed, adaptation to a low cAMP concentration would involve inactivation only of high affinity sites, leaving sites with lower affinities available to interact with cAMP at higher concentrations. However, the data shown in Fig. 6 (*inset*) make this unlikely. Consider that the loss of responsiveness to 10^{-8} M cAMP occurred more rapidly when cells were pretreated with 10^{-6} as compared with 10^{-7} M cAMP. The response to 10^{-8} M cAMP would presumably be mediated by receptors with a K_d no greater than 10^{-8} M cAMP. Because these high affinity sites would be saturated at either 10^{-7} or 10^{-6} M cAMP, inactivation should have occurred with equal rapidity. Similar arguments suggest that the adaptation effector does not directly inactivate adenylate cyclase (3).

Because adaptation does not appear to involve a direct effect on adenylate cyclase, the activity of this enzyme must be subject to transient positive modulation. In our scheme, this positive modulator, X, is the means by which the levels of excitation and adaptation are compared (6). The excitation and adaptation effectors might physically interact to form an inactive complex or compete for a common ligand. Alternatively, the effectors of excitation and adaptation might be enzymes that synthesize and degrade X. Receptor occupancy would determine the rate and extent of activation of these enzymes. Because excitation is activated more rapidly than adaptation, X is transiently elevated. Excitation and adaptation could also be mediated by membrane ion channels or pumps, the levels of reversible covalent modifications of macromolecules, or the levels of certain metabolites.

An adaptation mechanism has also been inferred from the behavioral responses of bacteria to chemoattractants (for reviews, see references 11 and 15). In the absence of attractants, bacteria alternate between episodes of smooth swimming and tumbling. An increment in the concentration of attractant suppresses tumbling for several minutes. A subsequent increment can elicit a second transient response. The duration of the response is proportional to the size of the increment in attractant receptor occupancy; the rate of adaptation is constant (1). In *D. discoideum*, however, the magnitude of the response

is proportional to the increment in cAMP binding and the duration of the response remains relatively constant; the rate of adaptation depends on the magnitude of the stimulus. Another major difference between the two response-adaptation systems is the rate of deadaptation. In bacteria, deadaptation occurs over seconds, whereas adaptation takes several minutes (1); in *D. discoideum*, the time-courses of adaptation and deadaptation (6) are similar, each with a $t_{1/2}$ of ~3 min. We infer from these characteristics that the mechanism of adaptation in these two organisms may be distinctly different.

Our physiological characterization of the cAMP signaling response should be useful in understanding the control of cell behavior by cAMP signals during aggregation. It is clear that cAMP signaling responses are neither stereotyped nor independent of the stimulus once initiated. There are no fixed thresholds for signaling; responses can be elicited against varied backgrounds of cAMP. There is no absolute refractory period after a signaling response, so that sequential responses can be elicited at various frequencies. The magnitude of a response depends both on the concentration of a current cAMP stimulus and the prior history of stimulation. The flexibility of the response-adaptation system might be important in the transfer of information between amoebae via the cAMP signaling response; cells may not just signal their presence but also transmit information concerning the shape and velocity of the propagated cAMP wave. A remaining challenge is to elucidate the molecular basis of adaptation in *D. discoideum* and to correlate the properties of the cAMP signaling response with the behavior of aggregating amoebae.

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