Cyclic 3′,5′-AMP Relay in *Dictyostelium discoideum* IV. Recovery of the cAMP Signaling Response After Adaptation to cAMP

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ABSTRACT In *Dictyostelium discoideum*, an increase in extracellular cAMP activates adenylate cyclase, leading to an increase in intracellular cAMP and the rate of cAMP secretion. Cells adapt to any constant cAMP stimulus after several minutes, but still respond to an increase in the concentration of the stimulus. We have now characterized the decay of adaptation (deadaptation) after the removal of cAMP stimuli. Levels of adaptation were established by the perfusion of [^{3}H]adenosine-labeled amoebae with a defined cAMP stimulus. After a variable recovery period, the magnitude of the signaling response to a second stimulus was measured; its attenuation was taken as a measure of residual adaptation to the first stimulus. The level of adaptation established by the first stimulus depended on both its magnitude and duration. Deadaptation began as soon as the first stimulus was removed. The magnitude of the response to the second stimulus increased with the recovery time in a first-order fashion, with a $t_{1/2}$ = 3–4 min for stimuli of 10^{-8} M to 10^{-5} M cAMP.

Responses to test stimuli, although reduced in magnitude, had an accelerated time-course when they closely followed a prior response that had not completely subsided. This effect is called priming; we believe it reveals a reversible, rate-limiting step that modulates the onset and termination of the signaling responses of amoebae that have not recently responded to a cAMP stimulus.

We have suggested that the cAMP signaling response is controlled by two antagonistic cellular processes, excitation and adaptation. The data reported here imply that both the rate of rise in the adaptation process and the final level reached depend on the occupancy of cAMP surface receptors and that the decay of adaptation when external cAMP is removed proceeds with first-order kinetics.

Dictyostelium discoideum amoebae communicate during aggregation through signals of extracellular cyclic adenosine 3′,5′-monophosphate (cAMP) that elicit both chemotactic movement toward the cAMP source and the secretion of additional cAMP. The *D. discoideum* signaling response can be elicited in vitro by exogenous cAMP stimuli. The binding of cAMP to cell surface receptors (8, 9) leads to activation of an adenylate cyclase (6, 10, 14) and an increase in intracellular cAMP, promptly followed by its secretion (6). When the extracellular stimulus is removed, cAMP dissociates from its binding sites with a $t_{1/2}$ of a few seconds (13), and intracellular cAMP rapidly declines to low, prestimulus values (6). However, even when a cAMP stimulus is held constant, the activation of adenylate cyclase is transient (6). Both intracellular cAMP levels and the rate of cAMP secretion peak after ~2 min of stimulation and then fall to basal values after 5–10 min (3, 4, 6). The mechanism by which amoebae terminate their signaling response to extracellular cAMP has been referred to as adaptation (4). This mechanism presumably serves to prevent cells from responding indefinitely to their own secretions, which would disrupt intercellular signaling. Adaptation appears to reflect the adjustment of cell sensitivity to the ambient concentration of extracellular cAMP in that adapted cells become unresponsive to a given cAMP stimulus, yet respond to further increases in the external cAMP concentration. Adaptation persists as long as extracellular cAMP is present, but can be reversed by removing the stimulus (4).

It is the magnitude of a signaling response, as measured by

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the total amount of cAMP released from the onset of the stimulus to the spontaneous termination of the response, and not its duration that is related to the increase in cAMP bound to surface sites (4, 8, 9). Once cells have adapted to a given stimulus, the response to an increment in extracellular cAMP is governed by the net increase in receptor occupancy and not its final level. The total amount of cAMP secreted during a series of consecutive stimulus increments is approximately equal to that elicited by a single stimulus of the highest concentration in the series (4). This phenomenon, called additivity, suggests that the magnitude of the response to each successive increment in the stimulus is limited by the preexisting level of adaptation.

Two process schemes have previously been used to describe the regulation of transient cellular responses to constant external stimuli, such as the motility of bacteria in the presence of chemoattractants (1, 12, 15). These concepts led to the development of a working hypothesis for analyzing the cAMP signaling response in *D. discoideum*. In our scheme, shown in Fig. 1, the occupancy of surface cAMP receptors controls the extent or level of both an excitation process and an adaptation process that counteracts excitation. The relative excess of excitation compared to adaptation at any instant is reflected in the value of a parameter, *X*, that determines the activity of adenylate cyclase. An increase in the extracellular cAMP concentration increases the extent or level of both excitation and adaptation. Both processes rise from prestimulus levels at a rate proportional to the increment in receptor occupancy, but the excitation level changes more rapidly than the level of adaptation. A transient response ensues, which terminates when the adaptation level matches the excitation level at the new value specified by receptor occupancy. Serial increases in cAMP receptor occupancy merely repeat this sequence of events; conversely, both excitation and adaptation decay when receptors are vacated. The magnitude of a signaling response is determined by the change in the levels of excitation and adaptation from their values at the onset of a stimulus to their final occupancy-specified value. The size of the response elicited by a single increase to a given concentration of cAMP equals that elicited by a stepwise increase to this same concentration. In the latter case, the response to an increment stimulus is appropriately scaled because amoebae keep track of the preceding cAMP stimulus, even when the response has ceased, through the altered levels of excitation and adaptation.

The control elements in this scheme, and adaptation in particular, have not been investigated directly. We therefore explored the dynamics of the adaptation process in this and a companion study (7). In this report, we characterized "deadaptation," the decay of adaptation upon removal of a cAMP stimulus, by examining the effect of a prior cAMP stimulus on the magnitude of the signaling response to a second stimulus administered after varied recovery intervals.

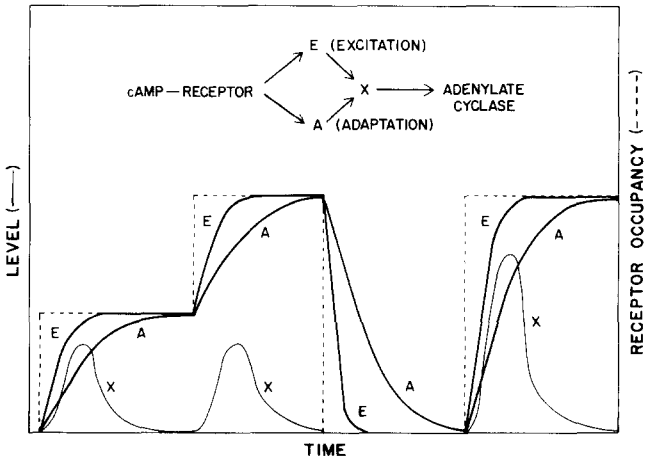

FIGURE 1 Excitation-adaptation scheme for control of the cAMP signalling response. Upper part of the figure indicates that cAMP receptor occupancy specifies changes in two hypothetical processes, excitation (*E*) and adaptation (*A*). *X* represents a process that continuously compares the levels of E and A and directly controls the activity of the adenylate cyclase. The lower portion of the figure shows a specific example. Dashed bars represent the occupancy of cAMP surface receptors. A small stimulus increases receptor occupancy from zero to O_1, leading to a rapid increase in *E* and a slower increase in *A*. *X* rises to a peak value and then declines as the value of *A* approaches that of *E*. A subsequent increment in receptor occupancy from O_1 to O_2 causes further increases in *E* and *A* and another transient rise in *X*. When the stimulus is removed, *E* (rapidly) and *A* (slowly) return to their basal values and sensitivity is regained. The final large increase in receptor occupancy from zero to O_2 elicits greater increases in both *E* and *A* and a greater transient change in *X*. The sum of the amount of cAMP production elicited by the two smaller increments equals that elicited by the single larger increment.

MATERIALS AND METHODS

Conditions for growth and development of the NC-4 strain of *D. discoideum* were as described in reference 6. Amoebae were fed [^{3}H]adenosine-labeled *Escherichia coli* for 3 h, transferred to nutrient-free agar plates at a density of 0.7–1 × 10^6 cells/cm^2, and incubated for 5–7 h at 22°C in the dark. At the first signs of aggregation, cells were harvested and replicate aliquots of 0.5–1.5 × 10^6 cells transferred to Millipore filters (SSWPO 1300; Millipore Corp., Bedford, Mass.) in a perfusion apparatus. To measure total radioactivity, the filters were placed in scintillation vials at the end of the experiment and counted in 1 ml of 1% Triton X-100 plus 7 ml Triton-toluene fluor (3).

All experiments were performed with single-, 4-, and 8-filter perfusion devices as described (3, 5, 6). Perfusion solutions were delivered via multichannel Gilson Minipuls pumps (Gilson Medical Electronics, Inc., Middleton, Wis.); the flow rates for all lines were nearly identical at 4.5–5.5 s/drop. Filter effluents were collected manually or with fraction collectors (model M75, Medical Research Corp., Boston, Mass.) equipped with photocell drop counters. Fractions of 6–24 drops (45 μl/drop) were collected in test tubes containing 20–30 μl of a phosphodiesterase stopping solution (0.2 M HCl, 50 mM dithiothreitol (DTT), and 10^{-3} M cAMP [3]).

All experiments were carried out at 19°–22°C. A standard routine was used to synchronize labeled amoebae after transfer to the perfusion apparatus (6). Freshly loaded cells were perfused with M-KK_2 buffer (6) for 8–15 min, then with 10^{-8} M cAMP for 3 min, and finally with M-KK_2 for an additional 15–20 min. The experiment was then initiated by administering cAMP stimuli.

Effluent fractions were processed immediately for [^{3}H]cAMP or frozen at −20°C. Each sample was neutralized with 5 μmol of Tris base, added in a 0.5–1-ml vol. [^{3}H]cAMP purification was accomplished by sequential chromatography on Bio-Rad AG 50WX-4 and Bio-Rad neutral alumina as described in reference 6. Recovery of [^{3}H]cAMP was 60–80%, with most of the loss occurring at the AG 50WX-4 step. The purified samples were processed for liquid scintillation spectroscopy as described (3) except that 1 ml of 1% Triton X-100 was used instead of 1 ml of 1% SDS to resuspend dried samples. This method of purification removed essentially all tritiated compounds secreted by amoebae, other than [^{3}H]cAMP. Only a counting background of 8–12 cpm has been subtracted in the data presented.

Experimental Strategy

A cAMP stimulus was administered to produce a certain level of adaptation in amoebae. The attenuation of the response to a second stimulus should be a measure of the residual level of adaptation created by the first stimulus, just as responses to consecutive stimulus increments are attenuated by adaptation to the preceding stimuli (4). The first stimulus was of any duration, whereas the second stimulus was maintained until the response was completed (5–10 min). The time-course of deadaptation was assessed by varying the recovery interval between the first and second stimuli.

The effect of the first stimulus on the magnitude of the response to the second stimulus was quantitated as a recovery ratio = (size of response to second stimulus)/(size of response to control stimulus). The total amount of [^{3}H]cAMP (cpm) secreted during the stimulus was used to quantitate the magnitude of the response. (This was assumed to represent a constant fraction of the total cAMP synthesized in response to the stimulus, with the remainder degraded intracellularly (6)). If the two paired stimuli were of identical concentration and duration, the amount of cAMP released during the first stimulus in the pair was used for the denominator. In other cases, the appropriate control response was evaluated by stimulating a duplicate filter of amoebae in parallel.

Normalization to Maximal Recovery and Variability in Recovery Ratios

Recovery ratios were routinely normalized so that, for a given pair of cAMP stimuli, the mean maximal recovery ratio = 1 (unless otherwise noted). The mean maximal recovery ratio was the average of all recovery ratios obtained at recovery intervals equal to or greater than that needed for maximal recovery, usually 15 min. The value of the recovery ratios and the time-courses of recovery varied somewhat from day to day. The standard deviation of the mean maximal recovery ratio was approximately ±20%. Within an experiment, responses of replicate filters to the same first stimulus rarely differed by more than 20% and recovery ratios obtained using the same stimulus pairs agreed within 10%.

RESULTS

Experiments in this study were designed to examine the effect of a prior cAMP stimulus on the response to a second, test stimulus. An example is shown in Fig. 2. In response to a 10^{-8} M cAMP stimulus, the rate of cAMP secretion rapidly increased to peak at ~2–3 min and then declined toward basal levels. When the stimulus was removed for 2 min, restimulation at the same concentration elicited a second response, indicating recovery from the adaptation that had terminated the first response. However, the second response was reduced in magnitude, compared to the first, suggesting that deadaptation was incomplete. As the recovery interval was extended in similar experiments, the second response increased to a maximum by 15 min (see below).

Irreversible Changes in the Signaling Response

The maximal response to a second stimulus generally did not return to that of the control even after 30–45 min of recovery. Responses elicited from amoebae perfused for 100 min with buffer alone were identical to those elicited by stimuli administered after 30 min. Thus, the failure to recover completely was attributable to an effect of the first cAMP stimulus rather than to deleterious effects of perfusion or to developmental changes during the interval separating the pair of stimuli.

The degree to which responsiveness was irreversibly reduced depended on the dose and duration of the first cAMP stimulus. Extending the duration of the first stimulus progressively diminished the maximal size of the response to a second stimulus of the same concentration (Fig. 3). This effect was more rapid when a saturating stimulus (10^{-5} M cAMP) was used, but even 10^{-8} M cAMP produced a persistent reduction in responsiveness. The ability to respond to low stimulus concentrations was more sensitive to irreversible attenuation by a preceding stimulus. Table I shows results from a representative experiment in which a 10-min stimulus of 10^{-8}, 10^{-7}, or 10^{-5} M cAMP was followed 30 min later by a test stimulus. The amount of cAMP secreted in response to a 10^{-8} M stimulus was reduced to 53% by a 10-min, 10^{-8} M cAMP stimulus given 30 min earlier. This same protocol reduced the response to 10^{-5} M cAMP by only 8%. Conversely, when a 10^{-8} M cAMP stimulus was preceded

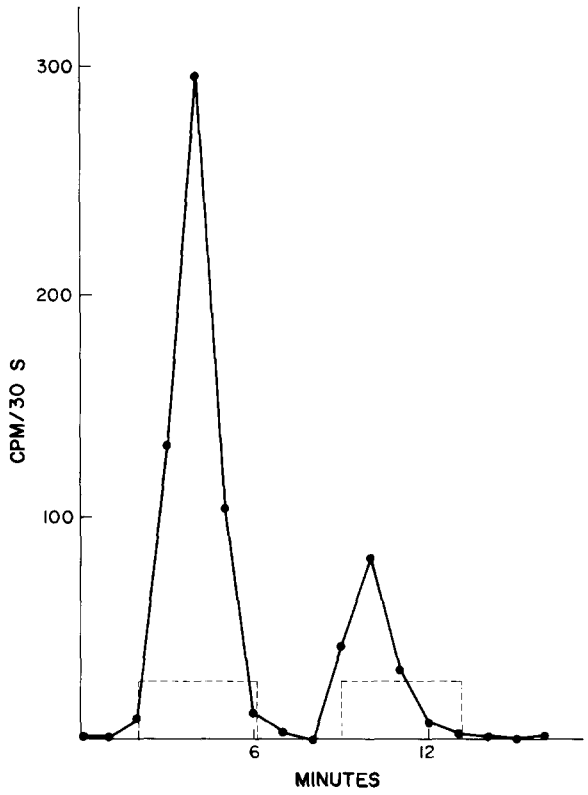

FIGURE 2 [^{3}H]cAMP secretion elicited by 10^{-8} M cAMP stimuli. Amoebae were stimulated with 10^{-8} M cAMP for 5 min, washed for 2 min, and then restimulated with 10^{-8} M cAMP for another 5 min, as depicted by the dashed lines. Fractions were collected each minute for [^{3}H]cAMP analysis. Total cell-associated radioactivity was 2.85×10^6 cpm/filter.

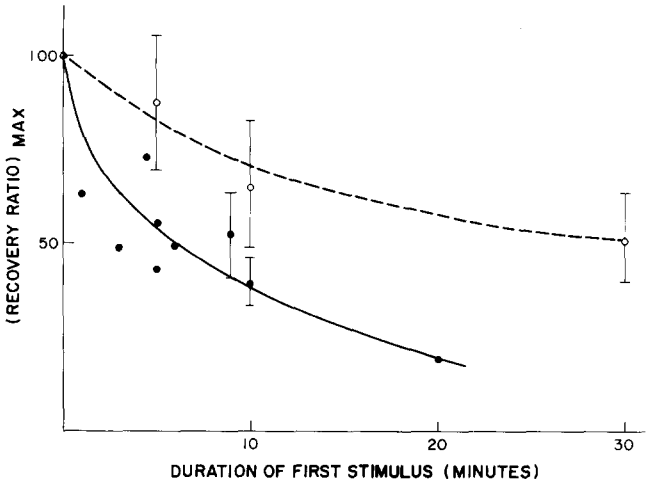

FIGURE 3 Irreversible reduction of the signalling response as a consequence of prior stimulation. Amoebae were perfused with pairs of stimuli of either 10^{-8} M cAMP (○) or 10^{-5} M cAMP (●). The first and second stimuli were separated by 15–45 min. Recovery ratios were not normalized. The mean maximal recovery ratio (*recovery ratio$_{max}$*) reflected the plateau value reached after recovery intervals of 15 min or more. The data with error bars are mean ± SD obtained from at least four experiments; the rest of the points are single determinations.

by a 10-min, 10^{-7} M cAMP stimulus, responsiveness was diminished to 34%, more than the effect produced by a preceding 10^{-8} M stimulus of the same duration.

Although the irreversible inhibition of the signaling response

TABLE I

Irreversible Reduction of cAMP Signaling Response at Different Test Stimulus Concentrations

	Second stimulus (recovery ratio)		
First stimulus	10^{-8} M cAMP	10^{-7} M cAMP	10^{-5} M cAMP
10^{-8} M cAMP	0.53	—	0.92
10^{-7} M cAMP	0.34 ± 0.07*	0.49	0.58
10^{-5} M cAMP	—	—	0.44

Except for *, the data were obtained in a single experiment, in which labeled amoebae were divided equally among eight filters, synchronized, and then presented with a pair of 10-min cAMP stimuli, 30 min apart. Recovery ratios were calculated as described in Materials and Methods, but were not normalized to the mean maximal recovery ratio.

* Mean ± SD for five separate determinations of the unnormalized recovery ratio obtained in experiments carried out similarly to that described above. Amoebae were treated with a 10^{-7} M cAMP stimulus for 10 min, perfused with buffer for 30 min, and tested with a 6-min, 10^{-8} M cAMP stimulus. The recovery ratio was calculated using the response elicited by a control 6-min 10^{-8} M cAMP stimulus delivered in parallel to an identical filter of amoebae.

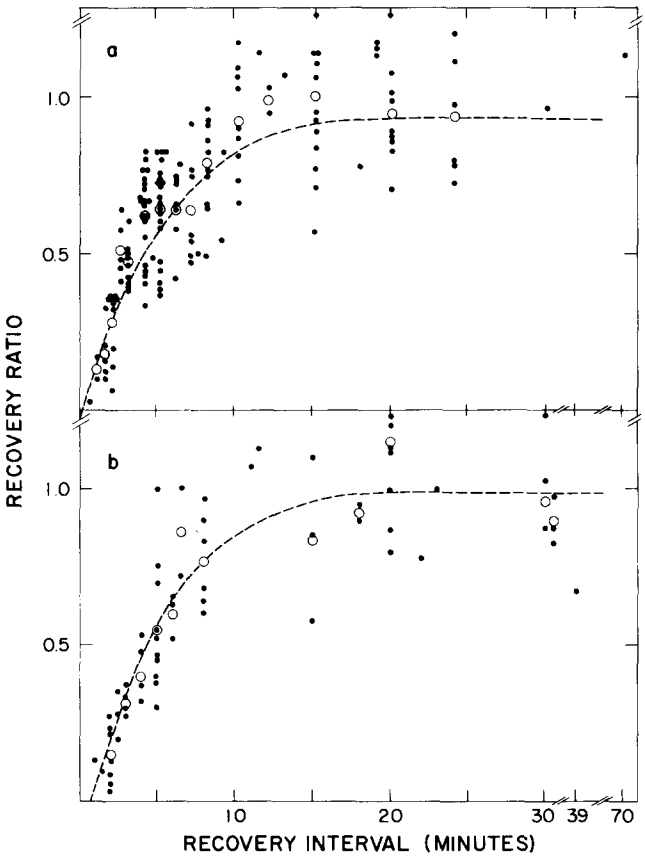

FIGURE 4 Recovery of responsiveness to 10^{-8} M cAMP after the removal of a 10^{-8} M cAMP stimulus. ●, Recovery ratios plotted as a function of the time interval separating a pair of 10^{-8} M cAMP stimuli. ○, Mean values of the recovery ratios for the recovery interval. Dashed line, fit of the data to a first-order exponential function, ln(1 − recovery ratio) vs. recovery interval. *(a)* Recovery from a 5-min 10^{-8} M cAMP stimulus. Results from 57 experiments. The unnormalized mean maximal recovery ratio for intervals ≥15 min was 0.88 ± 0.19 (n = 34). The points plotted above the slashes on the ordinate axis are 1.37 at 15 min and 1.27 at 20 min. *(b)* Recovery from a 10-min 10^{-8} M cAMP stimulus. Results from 26 experiments. The unnormalized mean maximal recovery ratio for intervals ≥18 min was 0.65 ± 0.17 (n = 20). The points plotted above the slashes on the ordinate axis are 1.73 at 20 min and 1.33 at 30 min.

might represent an independent process worthy of separate study, it complicated the assessment of deadaptation. It is the reversible changes in responsiveness that are likely to regulate signal relay in aggregating amoebae. To examine this component of recovery from cAMP stimuli, recovery ratios were normalized so that the maximal value was unity (see Materials and Methods).

Recovery from Stimuli of 10^{-8} M cAMP

The reversible attenuation of responsiveness produced by a 10^{-8} M cAMP stimulus of 5 min duration was monitored by restimulating with the same concentration after a variable recovery interval. The recovery ratio is plotted as a function of the recovery interval in Fig. 4*a*. The amount of cAMP secreted in response to the test stimulus increased continuously with recovery time. The test stimulus elicited a response even if the recovery period was only 30 s. There was thus no evidence for an absolute refractory period after adaptation to a stimulus of 10^{-8} M cAMP. Half-maximal recovery was observed after 3–4 min; by 15 min, the magnitude of the second response had reached a plateau of 88% of that elicited by the first stimulus. The decay of adaptation after the removal of the first stimulus could account for the increase in responsiveness to test stimuli.

The secretion of cAMP in response to 10^{-8} M cAMP nearly subsided after 5 min of stimulation (Fig. 2). We wondered if the extent of adaptation would continue to increase if the stimulus were extended beyond this time. In this case, the time-course of recovery from a longer stimulus might be different. Therefore, we compared the kinetics of recovery from 5-min (Fig. 4*a*) and 10-min (Fig. 4*b*) stimuli of 10^{-8} M cAMP. The initial rate of recovery was slightly slower after a 10-min stimulus than after a 5-min stimulus. Half-maximal recovery occurred after a 4–5 min interval and maximal recovery (to 65% of a control) after ~15 min. The recovery curve was slightly sigmoid. Nevertheless, the overall similarity between the time-courses of deadaptation after the removal of 5- and 10-min stimuli of 10^{-8} M cAMP suggests that little additional increase in adaptation occurred when the stimulus was applied for the additional 5 min.

Recovery from cAMP Stimuli of Higher Concentration

To establish whether the time-course of deadaptation was affected by the concentration of the first stimulus, pairs of 10^{-7} M cAMP stimuli were given, separated by a variable recovery interval. (The duration of the first stimulus was extended to 10 min so that the rate of cAMP secretion fell to the same relative extent that occurred after 5 min of 10^{-8} M cAMP [cf. Fig. 2, 8*a*, and 9].) The time-dependent change in the recovery ratio (Fig. 5*a*) was quite similar to that observed for 10^{-8} M stimulus pairs (Fig. 4). There was no period of absolute refractoriness to restimulation. Half-maximal recovery occurred after a 3–4 min recovery interval, and maximal recovery was attained when >15 min separated the two stimuli. The size of the second response after maximal recovery was reduced to 53% of the first.

We also examined recovery from a 10^{-5} M cAMP stimulus of 9 min duration, using test stimuli of 10^{-5} M cAMP. This cAMP concentration elicits a maximal cAMP signaling response. The time-course of recovery (Fig. 5*b*) was similar to that observed for 10^{-8} and 10^{-7} M stimulus pairs. The mean maximal recovery was 49%.

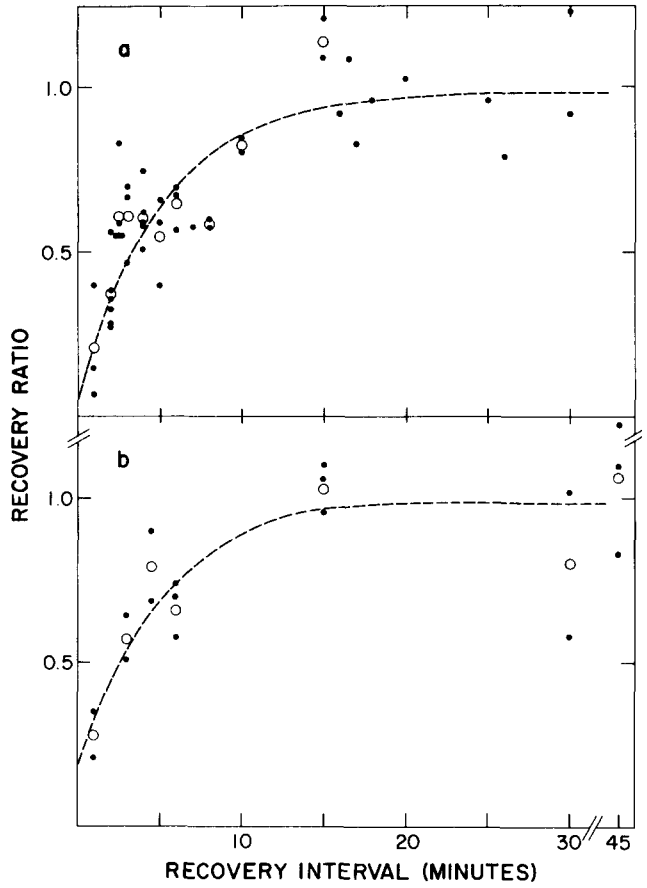

FIGURE 5 Recovery of responsiveness after the removal of a 10^{-7} M or 10^{-5} M cAMP stimulus. ●, Recovery ratios normalized to a mean maximal recovery ratio of unit. ○, means of recovery ratios. Dashed line, fit of the data to a first-order exponential function. *(a)* Recovery from a 10-min stimulus of 10^{-7} M cAMP, as tested by a second 10-min stimulus of 10^{-7} M cAMP. Results from 20 experiments. The unnormalized mean maximal recovery ratio for recovery intervals ≥15 min was 0.53 ± 0.07 (n = 12). *(b)* Recovery from a 9-min stimulus of 10^{-5} M cAMP as tested by a second 9-min stimulus of 10^{-5} M cAMP. Results from seven experiments are shown. The unnormalized mean maximal recovery ratio for recovery intervals ≥15 min was 0.52 ± 0.11 (n = 8). The point plotted above the slashes on the ordinate axis is 1.30 at 45 min.

Is Recovery a Change in the Sensitivity to cAMP Stimuli?

The extent of adaptation produced by cAMP stimulus appears to be proportional to its concentration (4). For example, a preceding stimulus of 10^{-7} M cAMP attenuates the response to a given increment in cAMP to a greater degree than a preceding 10^{-8} M stimulus. We propose that the time-dependent increase in the recovery ratio seen in Figs. 4 and 5 reflects a decay in adaptation after the removal of the first stimulus. If so, the recovery of responsiveness to a test stimulus should reflect the recovery of sensitivity to cAMP stimuli and depend on the relationship between the magnitude of the first and second stimuli. To test this premise, amoebae were stimulated for 10 min with 10^{-7} M cAMP, then tested with 10^{-8} M cAMP after a variable recovery interval (Fig. 6*a*, lower curve). An interval of ~14 min was required for half-maximal recovery, and maximal recovery occurred only after a 25- to 30-min interval. The prolonged course of recovery under these conditions was quite different from that observed with a test stimulus of the same concentration as the first (Figs. 4 and 5). For comparison, the recovery of responsiveness to a 10^{-8} M cAMP test stimulus that followed a 10-min, 10^{-8} M cAMP is included in Fig. 6*a*.

We also administered test stimuli of 10^{-7} M cAMP at various times after a 5-min, 10^{-8} M cAMP stimulus. As expected from previous results (4), giving the test stimulus without an inter-

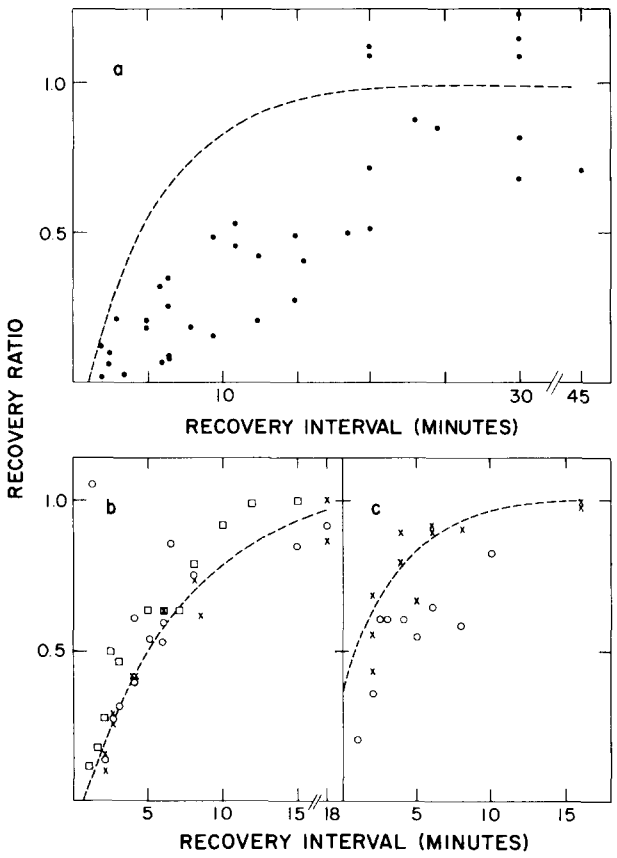

FIGURE 6 Dependence of the recovery of the signaling response on the concentration and duration of the first stimulus. *(a)* Recovery of responsiveness to 10^{-8} M cAMP after the removal of 10-min stimuli of 10^{-8} M or 10^{-7} M cAMP. ●, Recovery from a 5-min test stimulus of 10^{-8} M cAMP was tested at various times after the removal of a 10-min stimulus of 10^{-7} M cAMP. Results from nine experiments are shown. The unnormalized mean maximal ratio for recovery intervals ≥30 min was 0.34 ± 0.07 (n = 5). On any given day, the recovery ratio never increased beyond the value reached after 30 min of recovery. Dashed line, recovery of responsiveness to a 10^{-8} M cAMP test stimulus after the removal of a 10-min stimulus of 10^{-8} M cAMP, taken from Fig. 4 *b*. *(b)* Recovery of responsiveness to 10^{-8} M cAMP after the removal of 5- or 10-min stimuli of 10^{-8} M cAMP or 1.5-min stimuli of 10^{-7} M cAMP. *X*, Amoebae were tested with a 5-min stimulus of 10^{-8} M cAMP. Results from four different experiments are shown. The unnormalized mean maximal recovery ratio was 0.78 ± 0.09 (n = 3). Dashed line, fit of the data to a first-order exponential. The means of recovery ratios for test stimuli of 10^{-8} M cAMP after 5-min (□) or 10-min (○) stimuli of 10^{-8} M cAMP taken from Fig. 4 *a* and *b* are shown for comparison. *(c)* Recovery of responsiveness to 10^{-7} M cAMP after the removal of 1.5- or 10-min stimuli of 10^{-7} M cAMP. *X*, amoebae were stimulated for 10 min at 10^{-7} M cAMP at various times after the removal of a 1.5-min stimulus of 10^{-7} M cAMP. The recovery ratios from each of three experiments were normalized so that the mean maximal ratio, actually 0.96, was unity. Dashed line, fit of these data to a first-order exponential. ○: means of recovery ratios for pairs of 10-min stimuli of 10^{-7} M cAMP were taken from Fig. 5 *a*.

vening recovery period elicited a response that was 45 and 65% of the control response to 10^{-7} M cAMP in two separate experiments. When a 3-min interval separated the pair of stimuli, the magnitude of the response to 10^{-7} M cAMP was 84% of the control. Under identical conditions, however, the response to a test stimulus of 10^{-8} M cAMP was less than half-maximal (Fig. 4). Thus, after the removal of the first stimulus, substantially reduced responses were elicited by an identical test stimulus at times when almost full sensitivity to larger stimuli had returned.

Effects of Brief cAMP Stimuli on Subsequent Responses

If a stimulus is removed before the secretion of cAMP has spontaneously subsided, the magnitude of subsequent responses should be related to the adaptation level reached before the first stimulus was removed. Therefore, we examined the recovery of responsiveness to test stimuli of 10^{-8} M cAMP after a 1.5-min stimulus of 10^{-7} M (Fig. 6*b*). For comparison, we replotted the recovery of responsivness after 5- and 10-min stimuli of 10^{-8} M cAMP in Figure 6*b*. The effect of 1.5-min stimuli of 10^{-7} M cAMP appeared to be nearly identical to the longer stimuli of 10^{-8} M cAMP. In contrast, 10^{-7} M cAMP stimuli of 10-min duration caused a far more profound suppression of responses to 10^{-8} M cAMP (Figure 6*a*).

Stimulation with 10^{-7} M cAMP after a period of recovery from a 1.5-min stimulus of 10^{-7} M cAMP elicited larger responses than those measured after the same recovery interval after a 10-min stimulus (Fig. 6*c*). The greater degree of recovery after the brief stimulus is consistent with the notion that adaptation does not reach its maximal level after only 1.5 min of stimulation.

Time-course of Responses Elicited during the Recovery Period

We discovered that the time-course of secretion of cAMP in response to a test stimulus is characteristically altered by a prior cAMP stimulus. Fig. 7 shows the changes in the rate of cAMP secretion elicited by 10^{-8} M cAMP stimuli applied at various times after a 10^{-8} M cAMP stimulus of 5 min duration. When the recovery interval was short, the rate of cAMP secretion rose more rapidly during the second stimulus, peaked sooner, and declined earlier. Responses to test stimuli delivered after intermediate periods of recovery (4–6 min in Fig. 7) appeared to be compounded of both accelerated and normal kinetic curves. After a recovery interval of 15 min, the time-course of secretion during the test stimulus resembled that elicited by the first 10^{-8} M cAMP stimulus. Paired stimuli of higher cAMP concentrations exhibited similar features: upon restimulation after short recovery intervals, the time-course of cAMP secretion was shifted, but this acceleration disappeared as the recovery interval was increased. We shall refer to the acceleration of the time-course of cAMP secretion by a prior stimulus as priming.

The time-course of cAMP secretion during a second stimulus was accelerated even if the concentrations of the first and second stimulus differed. For example, a 5-min stimulus of 10^{-8} M cAMP primed the subsequent responses to 10^{-8} or 10^{-7} M cAMP stimuli given 4 min later (Fig. 8*a*). 5 min of stimulation with 10^{-6} M cAMP primed responses to 10^{-7} and 10^{-6} M cAMP test stimuli given after a 3-min recovery interval

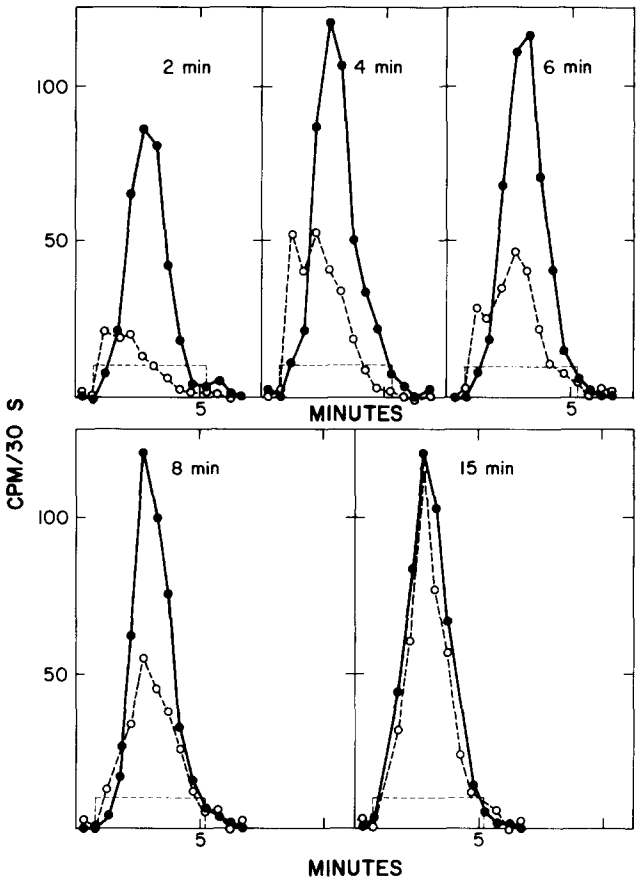

FIGURE 7 Time-course of [^{3}H]cAMP secretion elicited during recovery from a 5-min stimulus of 10^{-8} M cAMP. A 5-min stimulus of 10^{-8} M cAMP was delivered simultaneously to five filters of amoebae. After 2, 4, 6, 8, or 15 min of recovery, a second 5-min stimulus of 10^{-8} M cAMP was applied. Fractions were collected every 0.5 min and analyzed for [^{3}H]cAMP. Plotted are the respnse to the first ● and second ○ stimulus and the stimulus (dashed retangles). The recovery ratios for intervals of 2, 4, 6, 8, and 15 min were 0.28, 0.54, 0.50, 0.58, and 0.82, respectively (not normalized to mean maximal recovery).

(Figure 8*b*). Finally, 10^{-7} M cAMP was as effective as 10^{-6} M cAMP in causing acceleration of the response to a 10^{-7} M test stimulus (Fig. 8*b*).

Stimuli of brief duration also accelerated the time-course of responses to subsequent stimuli. In one such experiment, amoebae were stimulated for 0.5, 2, and 10 min with 10^{-6} M cAMP, and a second 10^{-6} M cAMP stimulus was administered after a 2.5-min recovery interval (Fig. 8*c*). The rate of cAMP secretion during the second stimulus was accelerated in all cases, independent of the duration of the first stimulus. In another experiment, 0.5 min of 10^{-8} M cAMP accelerated the response to a 10^{-7} M cAMP stimulus applied 2.5 min later.

Priming was not observed if the first stimulus was prolonged until the response to it disappeared completely. For example, when a 10^{-8} M cAMP stimulus was given for 10 min and followed by a 3-min recovery interval, a second 10^{-8} M cAMP stimulus did not elicit an accelerated response (Fig. 9*a*). Responses to high concentrations of cAMP have a more prolonged decline in the rate of cAMP secretion. A 10^{-7} M cAMP stimulus had to be extended to 20 min before priming disappeared; both 5- and 10-min 10^{-7} M cAMP stimuli accelerated the response to the test stimulus given 3 min later (Fig. 9*b*).

Priming was also observed in responses to increments in the

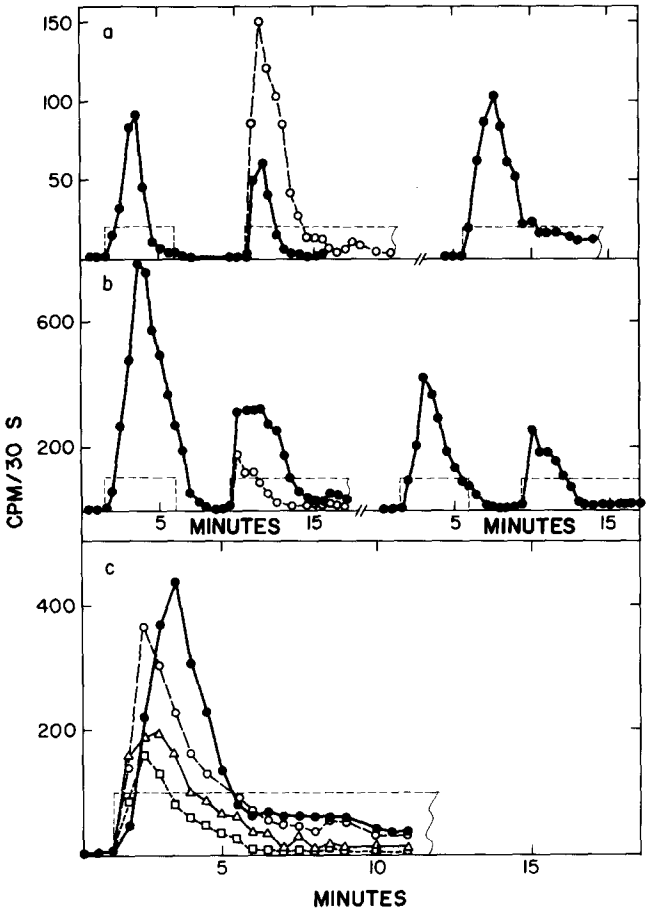

FIGURE 8 Priming of signaling response. cAMP stimuli were applied to filters of amoebae (dashed rectangles) and fractions were collected every 0.5 min for [^{3}H]cAMP analysis. Panels *a*, *b*, and *c* are results from separate experiments. *(a)* Left: ●, amoebae were stimulated with 10^{-8} M cAMP for 5 min, allowed to recover for 4 min, and then stimulated for 5.5 min with 10^{-8} M cAMP; ○, An identical filter of amoebae was stimulated in parallel with 10^{-8} M cAMP for 5 min (this response is not shown and was 1.15-fold larger than that illustrated for the ● filter), allowed to recover for 4 min, and then stimulated with 10^{-7} M cAMP for 9.5 min. Right: ●, a third filter of amoebae was stimulated in parallel for 10 min with 10^{-7} M cAMP for comparison. The magnitude of this response was 2.26 times larger than the mean response to the first 10^{-8} M cAMP stimulus. The recovery ratios for the test stimuli of 10^{-8} M and 10^{-7} M cAMP were 0.69 and 1.14, respectively (not normalized to mean maximal recovery). Total radioactivity associated with each filter was 2.2 × 10^6 cpm. *(b)* Left: ●, amoebae were stimulated with 10^{-6} M cAMP for 5 min, allowed to recover for 3 min, and then stimulated for 8 min with 10^{-6} M cAMP; ○, An identical filter of amoebae was stimulated in parallel with 10^{-6} M cAMP for 5 min (this response is not shown and was 78% of the response illustrated for the ● filter), allowed to recover for 3 min, and then stimulated with 10^{-7} M cAMP for 8 min. Right: ●, a third filter was stimulated in parallel for 5 min with 10^{-7} M cAMP, perfused with buffer for 3 min, and then restimulated for 8 min with 10^{-7} M cAMP. The magnitude of the response to the first 10^{-7} M cAMP stimulus was 53% of the mean response to the first 10^{-6} M cAMP stimuli delivered to the two other filters. The recovery ratios (not normalized to mean maximal recovery) were 0.51 for the pair of 10^{-6} M cAMP stimuli, 0.55 for the pair of 10^{-7} M cAMP stimuli, and 0.33 for the 10^{-7} M cAMP stimulus that followed a 10^{-6} M cAMP stimulus. Total radioactivity associated with each filter was 6.8 × 10^6 cpm. *(c)* Three filters of amoebae were stimulated in parallel with 10^{-6} M cAMP for 0.5, 2, or 10 min. The response to the 10-min stimulus is shown (●). After a 2.5-min recovery interval, a second stimulus of 10^{-6} M cAMP was applied for 10 min. The responses that followed a 0.5-min (○), 2-min (△), and a 10-min (□) stimulus are shown. The recovery ratios (not normalized to mean maximal recovery) were 0.88, 0.53, and 0.29, respectively. Total radioactivity asociated with each filter was 4.1 × 10^6 cpm.

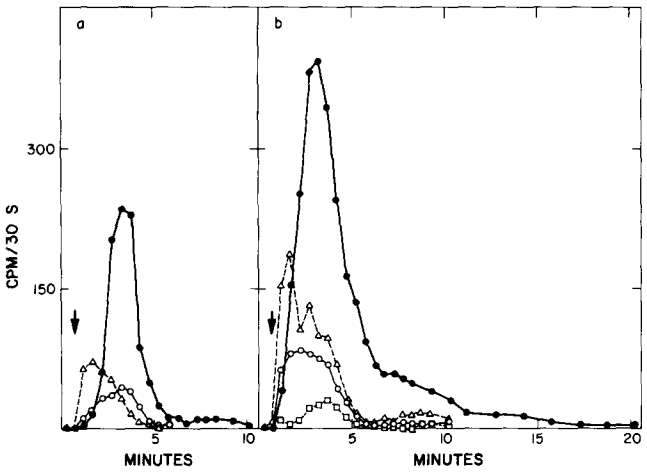

FIGURE 9 Effect of prolonged stimulation on priming of subsequent signaling responses. cAMP stimuli were applied to filters of amoebae (stimulus onset at the arrows) and fractions were collected every 0.5 min for [^{3}H]cAMP analysis. Total radioactivity associated with each filter was 3.3 × 10^6 cpm. *(a)* Amoebae were stimulated with 10^{-8} M cAMP for 10 min, and the response was measured (●). The cells were allowed to recover for 3 min and were then restimulated with 10^{-8} M cAMP for 5.5 min (○). A parallel filter received only a 5-min treatment with 10^{-8} M cAMP (the magnitude of the response was identical to that illustrated as ●), perfused with buffer for 3 min, and restimulated with 10^{-8} M cAMP for 5 min (△). Recovery ratios (not normalized to mean maximal recovery) were 0.36 and 0.24, respectively, for the responses following the 5- and 10-min stimuli. *(b)* Three filters of amoebae were stimulated in parallel with 10^{-7} M cAMP for 5, 10, or 20 min; the relative amount of [^{3}H]cAMP secreted during the first 5 min of stimulation was 0.82, 0.92, and 1.0, respectively. The response to the 20-min stimulus is plotted (●). After the first 10^{-7} M cAMP stimulus was removed, cells were washed with buffer for 3 min and then restimulated with 10^{-7} M cAMP for another 9.5 min. The response that followed the 5-min (△), 10-min (○), and 20-min (□) stimuli are shown superimposed. Recovery ratios (not normalized to mean maximal recovery) were 0.38, 0.24, and 0.06, respectively.

extracellular cAMP concentration, even though no recovery interval separated the first and second stimuli in these experiments (5). A low concentration of cAMP accelerated responses to subsequent large increments in cAMP concentration. When the duration of the first stimulus was prolonged beyond the cessation of the first response, however, the time-course of the response to a stimulus increment was significantly less accelerated.

DISCUSSION

An increase in extracellular cAMP elicits increased cAMP production and secretion in *D. discoideum* (3, 4, 6). Amoebae adapt to a constant stimulus after several minutes. The signaling response ceases because of a decrease in sensitivity (4). We have now characterized the kinetics of recovery of sensitivity to cAMP after the removal of a cAMP stimulus. During our investigation, we encountered an untoward complication (an irreversible decrease in responsiveness to cAMP) and a priming

phenomenon (acceleration of the time-course of signaling responses), both of which deserve comment.

The time-course of responses elicited by stimuli given either after short recovery intervals or by stimulus increments was accelerated. The accelerated course of cAMP secretion observed in responses to stimulus increments reflects a more rapid rise in the rate of cAMP production rather than an enhanced release of stored cAMP or a more rapid release of newly synthesized cAMP (6). Presumably, the primed responses elicited by stimuli that follow a preceding stimulus after a short recovery interval also share this feature.

Priming may be an all-or-none phenomenon. Even brief or small stimuli can prime responses to subsequent large test stimuli. The compound profiles in Fig. 7 suggest that cells pass rather discretely from the primed to the unprimed state after the removal of a cAMP stimulus, with a $t_{1/2} \cong 4$ min. A composite of the two overlapping time-courses could also explain the unusually broad secretion rate profiles of some of the primed responses.

The regulation of *D. discoideum* cAMP production presumably involves several steps between the binding of cAMP to surface receptors and the cellular processes that directly control the transient activation of adenylate cyclase. Priming of the response by a preceding stimulus might signify that an early step in this sequence is no longer rate-limiting. We lack an explanation for the disappearance of the priming effect when the first stimulus is prolonged beyond the termination of the signaling response. It is likely that other factors can also influence the kinetics of the signaling response. In some experiments, responses elicited from unadapted amoebae displayed a small accelerated component in their time-course (e.g., Figs. 2*b* and 3*c* in reference 7).

The magnitude of the response elicited by a test stimulus increased during the first 15–20 min of recovery from a prior cAMP stimulus but never returned to that elicited from control cells. The irreversible reduction in response size was dependent on both the dose and the duration of the first stimulus (Fig. 4 and Table I). A loss of functional cAMP binding sites may be involved. Klein and Juliani (11) have reported that prior exposure of amoebae to cAMP reduces the amount of [^{3}H]-cAMP binding at all [^{3}H]cAMP concentrations. In our studies, however, it seems as though responsiveness to low stimulus concentrations was more sensitive than responsiveness to saturating levels of cAMP. By analogy to the interpretation of the desensitization phenomenon in mammalian cells treated with hormones or neurotransmitters (2), the irreversible effects of cAMP stimuli could modulate the responsiveness of amoebae over long periods of time. However, the short-term, reversible modulation of responsiveness is more likely to be involved in the control of propagated intercellular cAMP signals during aggregation.

The present study provides a quantitative description of the recovery of responsiveness after prior stimulation with cAMP. This process, which we refer to as deadaptation, had these general features:

(a) The recovery of responsiveness involved a progressive increase in the sensitivity of cells to cAMP. The magnitude of a response to a test stimulus applied after a given recovery interval increased with the magnitude of the test stimulus.

(b) Deadaptation commenced without a lag as soon as a stimulus was removed; that is, there is no evidence for an absolute refractory period.

(c) Reduction of subsequent responses was significant after a 1.5-min stimulus of 10^{-7} M cAMP, suggesting that the process of adaptation commences earlier than the time the signaling response begins to decline (~2 min). This inference is more fully substantiated in the following report (7).

(d) The effect of a cAMP stimulus on the recovery of responsiveness depended on its concentration and duration. A brief stimulus of 10^{-7} M cAMP attenuated subsequent responses to 10^{-8} M cAMP to the same degree as a more prolonged stimulus of 10^{-8} M cAMP (Fig. 6*b*). Prolonged stimulation at 10^{-7} M cAMP retarded the recovery of responsiveness to 10^{-8} M cAMP as compared to recovery from a 10^{-8} M cAMP stimulus of the same duration (Fig. 6*a*).

(e) The level of adaptation evoked by a cAMP stimulus reached a plateau by the time the signaling response had nearly subsided. We infer this from Fig. 4, where the time-courses of deadaptation from 10^{-8} M cAMP stimuli of 5 and 10 min duration were found to be very similar. Apparently, little increase in adaptation occurred beyond 5 min of stimulation at this concentration. We suggest that the receptor occupancy-specified adaptation level is maintained until the stimulus is removed.

(f) The decay of adaptation proceeded with first-order kinetics with a $t_{1/2} = 3$–4 min. This conclusion is supported by the observation that the time-course of deadaptation was similar to stimuli of varied magnitude and duration and conformed well to a first-order exponential decay curve (5). The mechanism of deadaptation can thus be interpreted as a first-order decay in the level of adaptation from a value determined by the receptor occupancy and duration of the first cAMP stimulus.

The two-process scheme developed to account for the phenomenon of adaptation (Fig. 1) serves to summarize and

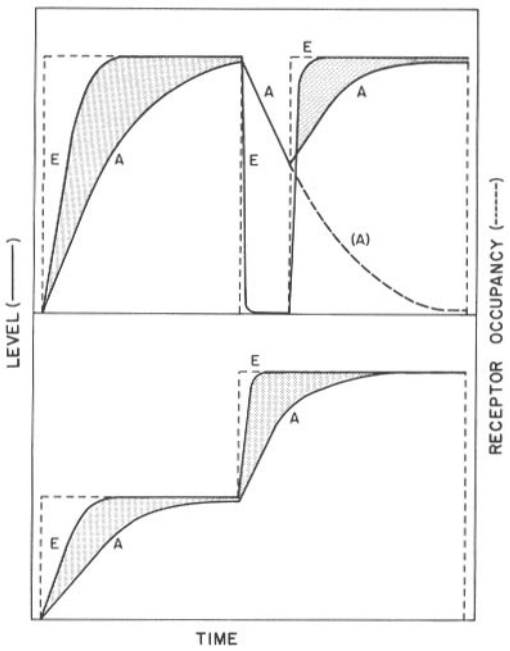

FIGURE 10 Application of excitation-adaptation scheme to cAMP signaling responses elicited after a prior priming stimulus. Dashed bars represent cAMP receptor occupancy. Shading is used to denote the signaling response when the excitation (*E*) level exceeds the adaptation (*A*) level. See text for discussion. *(top)* Hypothetical changes in *E* and *A* during paired stimuli separated by a brief recovery interval. *(bottom)* Hypothetical changes in *E* and *A* during a serial stimulus increment.

rationalize our observations on deadaptation, as illustrated in Fig. 10. The levels of excitation and adaptation rise with the onset of a cAMP stimulus to approach an occupancy-specified level. As shown in Fig. 10 (upper panel), removal of extracellular cAMP causes excitation to return quickly to its basal level, while the level of adaptation decays slowly. A second stimulus again drives the levels of excitaton and adaptation to an occupancy-specified value. Excitation must rise rapidly above the residual level of adaptation, because the ensuing response begins promptly. When the recovery interval was short, the time-course of the response was, in fact, accelerated. The priming effect is therefore denoted by a more rapid rise in both excitation and adaptation. In this scheme, the magnitude of a response elicited before adaptation has decayed completely depends on both the residual adaptation level and the cAMP receptor occupancy of the test stimulus. When amoebae are retested with the same cAMP concentration as the first stimulus (which would drive the levels of excitation and adaptation back to the same occupancy-specified value), the reduction of the test response reflects the fraction of the occupancy-specified level of adaptation still present at the onset of the second stimulus. The recovery of responsiveness as the interval between the two stimuli is increased reflects the time-course of deadaptation.

A larger second stimulus given during the deadaptation period would increase the levels of excitation and adaptation to a greater extent than a second stimulus of the same concentration as the first. This accounts for the finding that responses to larger test stimuli were relatively less affected by a residual level of adaptation. In contrast, the time-course of recovery to 10^{-8} M cAMP after the removal of a 10-min, 10^{-7} M cAMP stimulus was prolonged (Fig. 6*a*). In this case, a period of absolute insensitivity might have been expected, and no responses observed until adaptation decayed to below the maximal excitation level of the lower stimulus. However, responses to the smaller stimulus were detected even at short recovery intervals. Although this result is not entirely consistent with our simple model, deadaptation was clearly delayed.

The lower panel in Fig. 10 illustrates that the process of deadaptation studied here reflects the same cellular mechanisms as the phenomena of adaptation and additivity studied previously (4).

Previous studies on the *D. discoideum* signaling response showed that amoebae both respond and adapt to cAMP stimuli (3–5). In this investigation, we found that both the magnitude and the time-course of the signaling response depended in a characteristic way on the history of prior stimulation. Our results suggest that adaptation accumulates throughout a cAMP stimulus, reaching an occupancy-specific level that terminates a signaling response. Removal of the stimulus at any time allows the level of adaptation to decay with first-order kinetics. The magnitude of subsequent responses depends on the residual level of adaptation and the magnitude of the new stimulus. The regulation of *D. discoideum* signaling by an adaptation process dependent on both past and present stimuli would seem well-suited for signal processing and transmission during aggregation for morphogenesis.

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