CYCLIC 3′,5′ AMP RELAY IN *DICTYOSTELIUM DISCOIDEUM*

II. Requirements for the Initiation and Termination of the Response

PETER N. DEVREOTES and THEODORE L. STECK

From the Departments of Biochemistry and Medicine, The University of Chicago, Chicago, Illinois 60637

ABSTRACT

The secretion of ^{3}H-cyclic adenosine 3′,5′-monophosphate (cAMP) by prelabeled and suitably differentiated *Dictyostelium discoideum* amoebae was elicited in a perfusion apparatus by 10^{-10} to 10^{-5} M [^{14}C]cAMP stimuli of defined magnitude and duration. Exogenous stimuli evoked an immediate increase in the rate of [^{3}H]cAMP secretion which accelerated continuously to reach a peak of up to 100 times the unstimulated rate after 2–3 min of stimulation. Withdrawal of the stimulus at any time during the response led to a rapid decline to basal levels. Furthermore, a spontaneous decline in secretion rate was observed during prolonged cAMP stimulation, with a return to basal levels after 3–8 min of stimulation. After the initial secretory event, cells did not respond further to the continued presence of external [^{14}C]cAMP unless (*a*) it was interrupted by a brief recovery period or (*b*) the level of the stimulus was increased sufficiently. Since the second increment could follow the first at any time, continuous secretion of [^{3}H]cAMP could be sustained for up to 30 min by progressively increasing the stimulus between 10^{-10} and 10^{-5} M cAMP. The total magnitude of spontaneously terminated responses depended on the size of the increment in applied cAMP, larger stimuli evoking both a more rapid acceleration and a slower deceleration in [^{3}H]cAMP secretion rate. The integrated response to a given increment in stimulus level was apparently independent of its "shape"—i.e., the duration, magnitude, and number of sub-steps in the increment. These data support two mechanistic inferences: that amoebae respond in proportion to relative increases in extracellular cAMP concentration, but adapt to the concentration of cAMP itself. The data further suggest that the initiation and termination of the response are mediated by cellular component(s) beyond cAMP-occupied receptors.

KEY WORDS cyclic AMP · *Dictyostelium discoideum* · cellular slime molds · chemotaxis · adaptation · signal relay · intercellular communication

Suitably differentiated amoebae of the cellular slime mold, *Dictyostelium discoideum*, utilize secreted cyclic adenosine 3′,5′-monophosphate (cAMP) as a chemoattractant for aggregation into multicellular forms. Amoebae propagate this attractant by secreting cAMP in response to cAMP stimuli. This process, called cAMP relay, was first inferred from behavioral studies (2, 17, 21, 22) and has more recently been documented chemi-

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/79/02/0300-10$1.00
Volume 80 February 1979 300–309

cally (5, 6, 19, 24). It may be presumed to involve the binding of cAMP to cell surface receptors (9, 14), the activation of an adenylate cyclase (18, 20), an increase in intracellular cAMP (6), a secretion step, and, finally, a refractory period during which cells temporarily fail to respond to the presence of extracellular cAMP (7, 15, 23).

We now present a detailed description of the relay response of *D. discoideum* amoebae to stimuli of defined magnitude and duration, using the perfusion apparatus and techniques described in the preceding paper (4).

MATERIALS AND METHODS

Media and techniques employed were identical to those outlined in reference 4. Amoebae were fed [^{3}H]adenosine-labeled *Escherichia coli* for 1–3 h and allowed to develop on 2-cm^2 agar disks. In experiments illustrated in Figs. 1 and 3 and some of those in Fig. 4, amoebae were developed on Millipore filters (Millipore Corp., Bedford, Mass.). The mode of development was not critical to the main features of the response described herein. Labeled amoebae were harvested when the first signs of aggregation were visible (i.e., stippled aggregation [24]).

In every experiment other than those shown in Figs. 1 and 4 (see legends), [^{3}H]cAMP was purified by Dowex-50W chromatography (4). In some experiments, 50 μl (<10%) of the collected fractions was removed before neutralization to monitor total radioactivity.

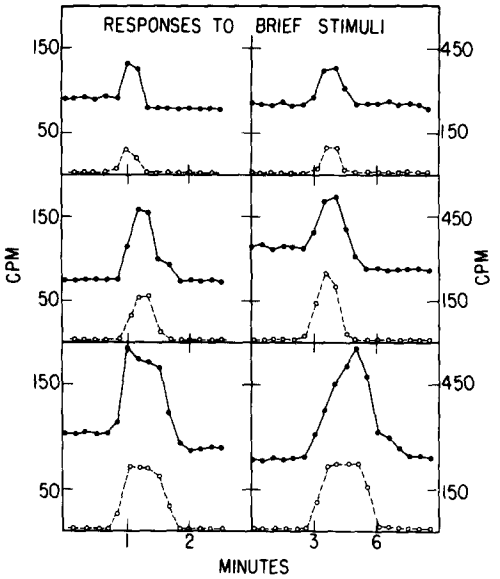

FIGURE 1 Secretory responses to brief [^{14}C]cAMP stimuli. NC-4 amoebae, labeled for 3 h, were allowed to develop on Millipore filters for an additional 6 h. Amoebae were washed with buffer for 15 min (not shown) and then perfused with 10^{-6} M [^{14}C]cAMP (--O--) at intervals. *Right*: three trials at a perfusion rate of 1 drop/7 s. *Left*: three subsequent trials at 1 drop/2.5 s. Stimuli were applied every 5–7 min; the entire experiment lasted ~50 min. Total tritium radioactivity is plotted (—●—) rather than [^{3}H]cAMP.

RESULTS

Relay Responses to Brief Stimuli (<3 Min)

Previous studies of cAMP relay in vitro failed to characterize the determinants of the duration and magnitude of this secretory response to cAMP stimuli. It is conceivable, for example, that responses are intrinsically regenerative (i.e., self-sustaining after initiation), "all-or-none," and of invariant magnitude and duration. The trigger of such an event could be a transient rise in extracellular cAMP above a fixed threshold or a sufficiently rapid increase in extracellular cAMP concentration (i.e., a high d[cAMP]/dt). We therefore studied the time course of cAMP secretion during brief stimulation.

As shown in Fig. 1, brief [^{14}C]cAMP stimuli elicited a concomitant increase in the rate of release of tritium, which we have previously demonstrated to be principally [^{3}H]cAMP (4). The secretory response rose promptly after the onset of stimulation and terminated rapidly after the stimulus was withdrawn. Secretion returned to the baseline rate with a half-time of less than 20 s. The elevated rate of secretion has never been observed to persist after removal of the [^{14}C]cAMP stimulus.

That the stimulus must be continuously present to sustain [^{3}H]cAMP secretion is further documented in the first 15 min of the experiment shown in Fig. 2. Amoebae were subjected to a train of 36-s stimuli, with an equal duration of buffer wash between stimuli. The amoebae responded to each [^{14}C]cAMP stimulus with an increase in their rate of [^{3}H]cAMP secretion which subsided following the withdrawal of exogenous cAMP.[1] These data demonstrate that cAMP relay responses to short stimuli were not fixed in dura-

[1] The response pattern illustrated in this experiment was consistently observed when amoebae were subjected to trains of brief (i.e., <1 min) stimuli separated by brief intervals. The second response was usually largest, and subsequent responses approached a low equilibrium value.

tion, but are dependent on the continued presence of cAMP in the surrounding medium.

Relay Responses to Prolonged Stimuli (>3 Min)

Stimulation of amoebae for prolonged periods demonstrated a different phenomenon (Fig. 3). The duration of the secretory response (i.e., the width at half-maximum secretion rate) was not proportional to that of the stimulus, but was limited instead to an interval of ~3 min. (The spontaneous termination of the response in the presence of a prolonged, constant stimulus has been previously observed by Gerisch and co-workers for stimulation with cGMP [7].) After the elevated secretion of [^{3}H]cAMP had subsided, no further secretory activity was observed for the duration of the stimulus, even when maintained for 19 min. When, after the initial 19-min exposure to 10^{-6} M cAMP, the stimulus was removed from the perfusing buffer for 8 min, a second relay response of similar form was elicited by the introduction of a second stimulus of 10^{-6} M cAMP.[2]

Figs. 1–3 were selected from numerous experiments relating the duration of the stimulus and the response. Fig. 4 summarizes the results of 27 independently initiated experiments, involving 74 individual responses to 10^{-6} M cAMP stimuli. There are two interesting aspects to this curve. The first is that the duration of the response approximately equals that of the stimulus up to ~3 min. Thereafter, the response time is independent of the duration of the stimulus. In one case, amoebae were continually stimulated for 2 h; after the initial response of 4 min, they did not respond again. In numerous other experiments, prolonged stimulation with cAMP at a concentration as low as 10^{-9} M elicited similar responses, i.e., a peak within 3 min followed by a cessation of secretion for the duration of the stimulus (up to

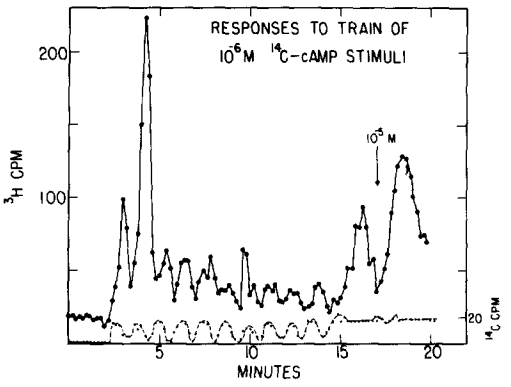

FIGURE 2 Serial [^{3}H]cAMP secretory responses to a train of brief [^{14}C]cAMP stimuli. NC-4 amoebae were labeled for 3 h, allowed to develop on agar for 8 h, and placed in the perfusion chamber and washed at a flow rate of 1 drop/4 s for 20 min. (The experiment shown was the third stimulation routine to which these amoebae were subjected; the first two, presented in Fig. 5, preceded this experiment by ~20 min of buffer wash.) In this experiment, a series of 10^{-6} M [^{14}C]cAMP stimuli, 36 s in duration, were applied at 36-s intervals. The experiment ended with a prolonged 10^{-6} M [^{14}C]cAMP stimulus, followed by raising the stimulus to 10^{-5} M with unlabeled cAMP (arrow). Each fraction (3 drops) was analyzed for [^{3}H]cAMP (see Materials and Methods). —●—, [^{3}H]cAMP; - -○- -, [^{14}C]cAMP.

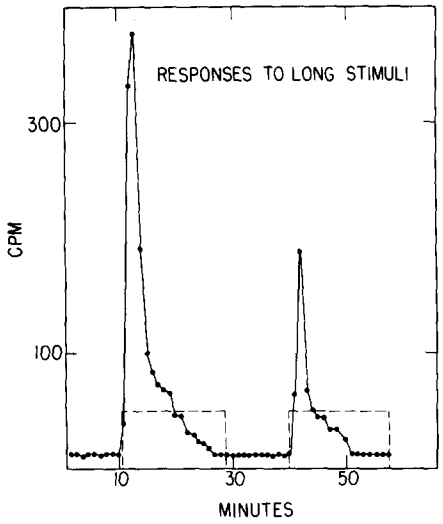

FIGURE 3 [^{3}H]cAMP secretory responses to prolonged, continuous cAMP stimuli. NC-4 amoebae labeled for 3 h were allowed to develop on Millipore filters for 7 h and washed with buffer for 25 min at a flow rate of 1 drop/10 s. Amoebae were perfused with 10^{-6} M cAMP as shown (dashed rectangles). 1-min fractions (6 drops or 300 μl) were collected for [^{3}H]cAMP analysis.

[2] A period of 8 min was usually sufficient to permit recovery of full relay activity when briefer stimuli were applied. As many as 18 similar responses have been elicited from the same preparation by brief stimuli delivered at 6-min intervals. The smaller second response in Fig. 3 may be a consequence of the prolonged initial stimulus at high concentration. Our preliminary results suggest that recovery time is a function of both the duration and concentration of the initial and secondary stimuli; this issue is under investigation (M. C. Dinauer, P. N. Devreotes, and T. L. Steck, unpublished data).

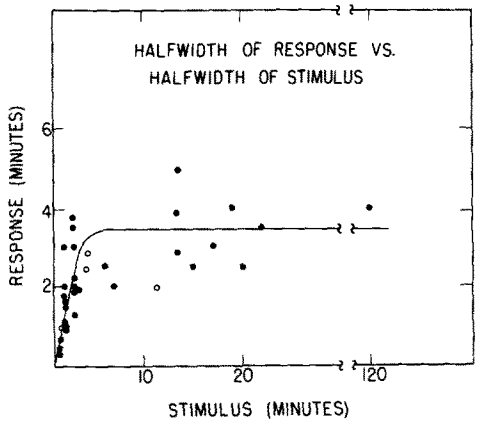

FIGURE 4 Dependence of the duration of the relay response on the duration of the cAMP stimulus. In a variety of experiments, in which different flow rates, developmental conditions, and stimulation routines were employed, cells were stimulated for various durations with 10^{-6} M cAMP and the response time was measured. Plotted is the width at half maximum of the applied [^{14}C]cAMP peak vs. the width at half maximum of the released [^{3}H]cAMP peak. In cases where unlabeled cAMP was applied, the length of application is reported. In experiments where released radioactivity was not analyzed for [^{3}H]cAMP, released [^{3}H]cAMP was estimated from the increase in the rate of release of total radioactivity over background rates (see reference 4). In experiments where the application of cAMP was longer than 4 min, released radioactivity was analyzed for [^{3}H]cAMP by Dowex 50W chromatography. Many points are superimposed on the graph. ●, NC-4; ○, AX-3.

25 min). An example of this behavior is discussed below (Fig. 5).

We have observed comparable behavior in the *D. discoideum* strain AX-3, in *D. purpureum* and under a variety of developmental conditions. In general, the responses of AX-3 amoebae are briefer, although larger, than those of NC-4 (4).

Detailed Analysis of the Time Course of the cAMP Relay Response

To elucidate the dynamics of the relay process, we followed in detail the time course of relay responses to prolonged cAMP stimuli. We perfused at high flow rate (1 drop/4 s) with maximal (10^{-6} M) and submaximal (10^{-9} M) [^{14}C]cAMP stimuli (see below). As previously demonstrated (4), the rapid perfusion technique minimizes the impact of both signal destruction (via secreted and cell surface phosphodiesterases) and signal amplification (via the local discharge of cAMP during relay) on the elicited response.

The data from this experiment are plotted in Fig. 5 in two forms: as [^{3}H]cAMP secretion per fraction (left) and as the integrated response (right). During stimulation with 10^{-9} M or 10^{-6} M cAMP, the rate of [^{3}H]cAMP secretion increased immediately, accelerated geometrically or exponentially for ~1 min, and then continued to accelerate linearly for nearly another minute (Fig. 5, left). While the initial time course did not differ significantly between the 10^{-9} M and 10^{-6} M cAMP curves, the linear acceleration was approximately four times greater at the high stimulus, contributing to a much enhanced total response (Fig. 5, right). (The maximal secretion rate observed in Fig. 5 was ~30-fold greater than the basal rate; in other experiments, this value has ranged from 6- to 100-fold.)

After ~2 min of continuous stimulation with either 10^{-9} M or 10^{-6} M [^{14}C]cAMP, the rate of [^{3}H]cAMP secretion abruptly decelerated without ever reaching a plateau, giving the profiles a "sawtooth" or spiked appearance (Fig. 5, left). The response to the 10^{-9} M cAMP stimulus declined by 50% in ~30 s and returned to the basal value within 3.5 min. The response to 10^{-6} M required nearly 2 min to decline by 50%. (This prolonged fall was the second factor contributing to the

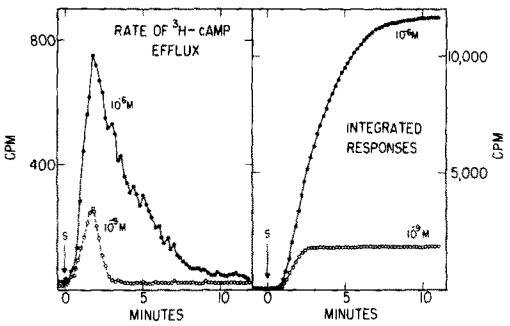

FIGURE 5 Comparison of responses to 0-10^{-9} M and 0-10^{-6} M cAMP concentration steps. See Fig. 2 for details of preparation of amoebae. *Left*: Rate of [^{3}H]cAMP release induced by 0-10^{-9} M (- -○- -) or 0-10^{-6} M (—●—) concentration steps. *Right*: Integrated responses calculated from rate data. (Baseline rate was subtracted from each fraction.) Arrow (S - stimulus) corresponds to the first fraction in which [^{14}C]cAMP could be detected. ([^{14}C]cAMP collected in each fraction after that was constant.) The stimulus of 10^{-9} M preceded that of 10^{-6} M, each lasted 16 min, and the two were separated by 20 min of buffer wash. Fractions of 3 drops (12 s) were collected and analyzed for [^{3}H]cAMP.

eightfold difference in total secretion observed between the 10^{-6} M and 10^{-9} M [^{14}C]cAMP-treated cells.)

The decline in the rate of [^{3}H]cAMP secretion elicited by 10^{-6} M cAMP appeared, in Fig. 5 and in other experiments, to be composed of rapid and slow components. Sometimes, the rapid phase dominated the deceleration profile (e.g., Figs. 3 and 8); other times, the slow phase dominated. In a variety of experiments (not shown), the rapid component reached the half-maximal value in 40–70 s and represented 50–80% of the total, while the slower component dropped to a half-maximal value in 3–5 min. In some examples (not shown), minor shoulders were apparent on the rising or falling limbs of the secretion profile (M. Dinauer, personal communication).

A Dose-Response Curve

Thus far, we have delineated several variables which influence the magnitude of the relay response to cAMP in our system. Among these are phosphodiesterase activity, autocatalytic self-stimulation, and the rate of perfusion (which affects the first two) (4). Furthermore, the duration of the stimulus is important, since secretion rates are not linear in time. (Thus, for example, responses to 10^{-9} M and 10^{-6} M cAMP in Fig. 5 were quite similar during the first 45 s of stimulation but were eightfold different overall.) In the light of these complexities, we attempted to construct a meaningful dose-response curve. We utilized rapid perfusion to "clamp" the stimulus concentration at the selected values (4). Stimuli of 8 min permitted the responses to run to completion spontaneously.

As shown in Fig. 6, responses increased with the size of the stimulus over a broad range. Stimuli of $<10^{-9}$ M cAMP elicited responses; saturation occurred near 10^{-5} M cAMP, and half-saturation occurred at 5×10^{-8} M cAMP.

Fig. 6 also demonstrates the impact of slow perfusion in the presence of DTT on such measurements. The relative response to low levels of cAMP was significantly increased, although maximal responses were not. We ascribe this effect to auto-catalytic magnification of the input stimulus (4). (Presumably, responses to low levels of cAMP would be even larger if flow were halted altogether.)

Relative Adaptation

As discussed at the conclusion of this report, the spontaneous cessation of secretory responses

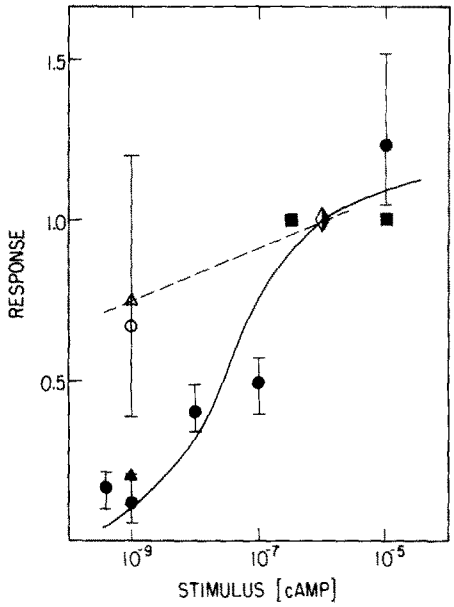

FIGURE 6 Dose-response curves for concentration steps originating at zero. Data from several experiments (different symbols) were combined by normalizing to responses to 0–10^{-6} M steps. Responses were total [^{3}H]cAMP released within 8 min of stimulus application. Error bars indicate range of integrated responses. Closed symbols represent responses monitored at flow rates of 1 drop/4–5 seconds; open symbols represent responses monitored at 1 drop/30 s in the presence of 2 mM DTT.

during prolonged stimulation can be regarded as a manifestation of adaptation to the stimulus. It was important to determine whether adaptation to a stimulus was relative. That is, could adaptation be overcome, without a recovery period, by simply raising the extracellular cAMP level? The experiment shown in Fig. 7 addressed this question. Amoebae were exposed to 10^{-9} M cAMP for 15 min. As before (e.g., Fig. 5), only an initial rise and spontaneous fall in [^{3}H]cAMP secretion rate were observed during this interval. Then, the cAMP concentration was increased to 10^{-6} M without a recovery period. A large response immediately ensued, followed, as before, by a decline in [^{3}H]cAMP secretion despite continued stimulation. A 15-min hiatus permitted a recovery of sensitivity, since renewed stimulation with 10^{-6} M cAMP then evoked a large secretory response.

We have characterized the adaptation process more fully in related experiments. An uninterrupted series of prolonged (14 min) stimuli were given in tenfold steps between 10^{-9} and 10^{-6} M cAMP. A secretory response, followed by spon-

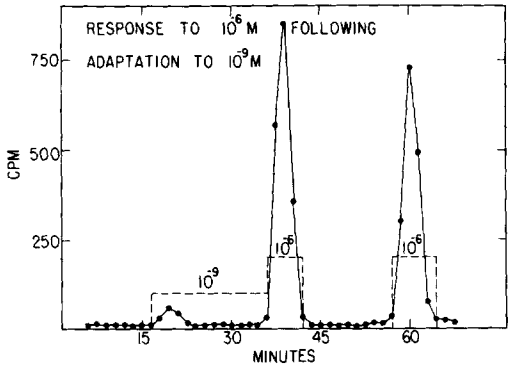

FIGURE 7 Response to 10^{-6} M after adaptation to 10^{-9} M. After labeling for 2.5 h, NC-4 amoebae were allowed to develop on agar for 8 h, and washed at 1 drop/5 s for 20 min. Stimulation with 10^{-9} M unlabeled cAMP followed by a direct switch to 10^{-6} M cAMP was as indicated by the dashed lines. 8-drop (400 μl) fractions were collected and analyzed for [^{3}H]cAMP.

taneous cessation, was elicited at each step, without the need for intervening recovery periods. This effect is also seen in the last several minutes of the experiment in Fig. 2. Fivefold increments in [^{14}C]cAMP also elicited relay responses from fully adapted cells. Similar results were obtained using the AX-3 strain of *D. discoideum*.

Elicitation of Prolonged [^{3}H]cAMP Secretory Responses

If the spontaneous fall in secretory rate during a prolonged cAMP stimulus is the result of relative adaptation, then cessation of the response might be forestalled by increasing the concentration of the cAMP stimulus during the adaptation period. This premise was substantiated in two types of experiments.

In the first, shown in Fig. 8, the [^{14}C]cAMP concentration in the perfusion buffer was increased from 0 to 10^{-6} M in four tenfold steps at 225-s intervals without intervening recovery periods. The response of [^{3}H]cAMP took the form of four peaks (i.e., adaptation occurred at each dose level) and the secretion of cAMP remained elevated above the basal level for >17 min. The abrupt steps in the profile of [^{14}C]cAMP show that there is little spill-over between fractions, so that the persistence of [^{3}H]cAMP between peaks cannot be ascribed to contamination by the peak material. Furthermore, an even more continuous (12 min) secretion of [^{3}H]cAMP was evoked by the second set of stepped stimuli (Fig. 8, 70–85 min), presumably because the increments were spaced 135 s apart and allowed little time for adaptation to reduce the secretion rate. Between the two trains of stimuli, a response to 10^{-6} M [^{14}C]cAMP was recorded for calibration and comparative purposes.

An even more effective means of eliciting prolonged [^{3}H]cAMP secretion was to administer a finely subdivided, discontinuous, geometrically-rising gradient of cAMP. Specifically, the cAMP concentration was doubled each 90 s over a 10^8-fold range from 10^{-13} to 10^{-5} M. As shown in Fig. 9, this treatment enhanced the secretion of [^{3}H]cAMP, most rapidly during stimulation with 10^{-9} to about 5 × 10^{-6} M cAMP. While the integrated response to a 0-10^{-5} M step was complete within 8 min (see also Fig. 5), the response to the geometrically-rising gradient persisted for ~30 min.

The Magnitude of Prolonged Responses

The magnitude of the total response to consecutive increments of stimulus could depend on the number, the relative size, and/or the duration of the increments. In that case, the magnitude of the response could vary widely for complex stimuli. Alternatively, the total response could be simply a function of the initial and final stimulus concentrations (C_2–C_1) and be independent of the aforementioned variables. In this case, the sum of the responses to a multistep stimulus would equal the

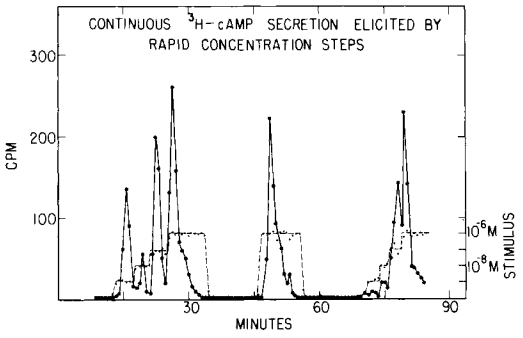

FIGURE 8 Continuous [^{3}H]cAMP secretion elicited by increasing concentration steps of [^{14}C]cAMP. After labeling for 2.5 h, NC-4 amoebae were allowed to develop on agar for 8 h, and washed at 1 drop/5 s for 20 min. [^{14}C]cAMP stimuli contained 10^{-9} M, 10^{-8} M, 10^{-7} M, or 10^{-6} M unlabeled cAMP and ~200, 400, 800, or 200 cpm/ml [^{14}C]cAMP, respectively. The dashed lines indicating the time courses of stimulus switches were calculated from the [^{14}C]cAMP eluting from Dowex columns. 10 drop fractions were collected. —●—, [^{3}H]cAMP; - - · - -, [^{14}C]cAMP.

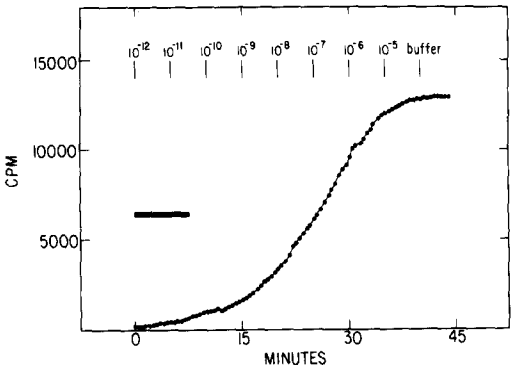

FIGURE 9 Continuous [^{3}H]cAMP secretion elicited by stimulation with a gradient of cAMP. After labeling for 2.5 h, NC-4 amoebae were allowed to develop on agar for 8 h. Amoebae were washed with buffer at a flow rate of 1 drop/5 s for 20 min before the portion of the experiment shown. During this time they were stimulated twice with 10^{-9} M cAMP and observed to respond. Amoebae were then stimulated with a geometric gradient of cAMP formed by 25 serial twofold dilutions of 10^{-5} M unlabeled cAMP. Each concentration increment was applied for 90 s; the final 10^{-5} M was applied longer as indicated. Plotted is the integrated response (baseline rate was subtracted from each fraction). After this stimulus, and after 20 min of buffer wash, amoebae were stimulated with an instantaneous 0-10^{-5} M step. The total duration (~8 min) of the response to this single step is indicated by the heavy bar. Six-drop fractions were collected, and analyzed for [^{3}H]cAMP.

magnitude of the response to the corresponding single-step stimulus. This concept is generally termed additivity.

We tested these possibilities by comparing integrated responses to pairs of single-step and multistep stimuli of the same total concentration span. We employed many different forms of multistep stimuli in these experiments (Table I). The validity of this approach depended on (*a*) maintaining the continuity of the stimulus (i.e., no interruptions or recovery periods) and (*b*) allowing the last response to proceed to spontaneous termination. The results were expressed as the ratio of the responses to complex and single-step stimuli. If the individual responses to the increments in the multistep stimulus were additive, this ratio would be unity. In nine experiments, the ratios fell between 0.5 and 2 with a mean near 1.

Variability in the Relay Response

While secretory responses to a given cAMP stimulus have reproducible time courses, the total amount of cAMP released can vary considerably. Many factors contribute to these variations. As demonstrated previously (4), developmental age, perfusion rate, phosphodiesterase activity, and autocatalytic signal amplification all influence the magnitude of the response. In the present paper, we have shown the response to be dose-dependent. We have also presented evidence for an adaptation mechanism which could cause relay responses to stimuli that follow closely after the termination of a preceding stimulus to be attenuated; this could be the cause of diminished responses seen in Fig. 3. Furthermore, Klein and Julian (12) have presented evidence for the loss of cAMP binding sites after prolonged exposure to high concentrations of this nucleotide; this effect could reduce responses to subsequent stimuli.

DISCUSSION

Our observations indicate that amoebae of *D. discoideum* respond to the increment in extracellular cAMP concentration (C_2–C_1), adapt within minutes to the new cAMP concentration (C_2), and remain adapted as long as that level of cAMP persists.

What is required of a stimulus to evoke a relay response? It is not simply elevation of extracellular cAMP above a fixed threshold level (3), since a fixed threshold can be exceeded only once by a complex stimulus. As shown in Figs. 8 and 9, repeated responses were elicited by multistep stimuli. Likewise, the response is not merely to the rate of change of the stimulating cAMP concentration, dC/dt. The cellular secretion rate continues to rise after dC/dt falls to zero during a "square-wave" stimulus (Figs. 3 and 5). Furthermore, very slow (multistep) and very rapid (single-step) jumps from C_1 to C_2 elicit the same total amount of cAMP release (Table I). It cannot be that the response, once triggered, continues to rise because it is stereotyped and indifferent to the stimulus, since withdrawal of the extracellular cAMP leads to a prompt cessation of the response (Figs. 1 and 2). Finally, responses required no minimum duration of cAMP contact; our briefest stimuli elicited [^{3}H]cAMP secretion (Figs. 1 and 2). Instead, the feature of the stimulus to which amoebae apparently respond is the increment in extracellular cAMP (C_2–C_1). Increments as small as twofold evoked readily detected responses (Fig. 9).

Two lines of evidence suggest that amoebae respond to extracellular cAMP increments in a

TABLE I

A Comparison of Responses to Single-Step and Multistep Stimuli

Description of stimulus pairs	Time to reach final concentration*	Multistep response / Single-step response
1. (4,10-fold steps to 10^{-6} M; 14 min per step)/(single step to 10^{-6} M)	42	1.68
‡2. (concave gradient to 2×10^{-7} M)/(single step to 10^{-6} M)	29	0.57
§3. (25,2-fold steps to 10^{-5} M; 90 s per step)/(single step to 10^{-5} M)	36	0.59
§4. (4,10-fold steps to 10^{-6} M; 225 s per step)/(single step to 10^{-6} M)	11.25	1.97
§5. (4,10-fold steps to 10^{-6} M; 135 s per step)/(single step to 10^{-6} M)	6.75	1.25
6. (6,5-fold steps to 10^{-6} M; 400 s per step)/(single step to 10^{-6} M)	33.33	0.69
‖7. (4 min 10^{-7} M, 8 min 10^{-5} M)/(single step to 10^{-5} M)	4	0.87
8. (12,3-fold steps to 10^{-5} M, 20 s per step)/(single step to 10^{-5} M)	3.67	0.71
¶9. (12,3-fold steps to 10^{-5} M, 20 s per step)/(single step to 10^{-5} M)	3.67	1.58

Eight of the nine experiments involved multistep and single-step stimuli, both originating at 0 and ending at the same cAMP concentration. (In experiment 2, the final concentration of the single-step exceeded that of the multistep.) The integrated responses to the multistep stimulus and the corresponding single-step stimulus were calculated and appropriate background values were subtracted from each (assuming that the low background secretion rate continued during the response) before calculation of their ratios.

* Interval (min) from start of the first increment to the start of the last.

‡ A concave gradient was formed as follows. The output of a sucrose gradient maker, containing 3 ml of 5×10^{-7} M cAMP in NFB in one chamber and 3 ml of NFB in the other, was pumped into a beaker initially containing 10 ml of NFB. Two lines pumped solution out of this beaker such that solution was removed twice as rapidly as it entered. One of these lines delivered solution to the cells. In test experiments employing [^{3}H]cAMP, the [^{3}H]cAMP concentration was observed to rise at an increasingly rapid rate from 0 to 2×10^{-7} M cAMP.

§ These experiments are from Fig. 8 and 9.

‖ Compares average of 2 multisteps to average of 3 single-steps.

¶ Compares multistep to average of 2 single-steps.

graded rather than regenerative, "all-or-none," fashion. First, when the extracellular cAMP concentration is controlled (as intended in using a perfusion system), the magnitude of a relay response is a function of the size of the stimulus; that is, submaximal cAMP increments elicit submaximal responses (Fig. 6). Second, the duration of cAMP secretion depends on the continued presence of extracellular cAMP. Removal of the stimulus at any time terminates the response promptly (Figs. 1 and 2).

The form of the response to an increment in external cAMP is a prompt increase in intracellular cAMP (4) and in the rate of [^{3}H]cAMP secretion which lasts about 2 min. Since this response occurs even under conditions where autocatalytic feedback loops should have no impact (i.e., rapid perfusion or stimulation with saturating cAMP concentrations [Fig. 5]), we conclude that the acceleration in secretion rate is an intrinsic feature of the response. Accelerating kinetics cannot be explained merely by a single first-order process, such as cAMP binding to surface receptors, since these processes decelerate in time. The initial geometric or exponential rise in the rate of cAMP accumulation (4) and secretion (Fig. 5) suggests that a multi-step pathway is involved.

What is the mechanism of relay response termination (i.e., return of the [^{3}H]cAMP secretion rate to prestimulus levels) in the presence of a constant stimulus? (*a*) A simple refractory (i.e., insensitive) period programmed by the cell (3) is

unlikely, since amoebae never respond a second time to the presence of a continuous stimulus but respond immediately, at any time, to a sufficient cAMP increment (Fig. 8). (*b*) Depletion of a critical metabolite, such as ATP (8), cannot be invoked as a termination mechanism because small responses are extinguished at least as rapidly as large ones (Fig. 5). Furthermore, a second response can always be elicited by a cAMP increment immediately after the first is fully attenuated (Figs. 7 and 8). (*c*) Feedback inhibition by intracellular cAMP or inhibition by a process which depends on intracellular cAMP (7) is not the mediator of response termination, since intracellular cAMP levels fall to basal values during prolonged stimulation without further relay response (Fig. 4 in reference 4 and unpublished observations). (*d*) Termination of secretion is not likely to involve "desensitization" or "down regulation" (10–12, 16). In such a mechanism, functional binding sites disappear after their interaction with the effector molecule. This process should occur more rapidly at higher levels of stimulus. However, if anything, termination occurred earlier at lower stimulus concentrations (Fig. 5). Furthermore, repeated responses can be initiated without a recovery period.

Our data on the termination of the relay response are most compatible with an adaptation mechanism. By adaptation, we mean the reversible extinction of responsiveness caused by the adjustment of cell sensitivity to the level of the stimulus. This explains why complete cessation of a relay response occurs in the presence of a constant, prolonged stimulus and why no recovery of responsiveness can be detected as long as the stimulus is held constant (Figs. 3, 4, and 5). It accounts for recovery of full sensitivity ("de-adaptation") after removal of the stimulus (Figs. 3, 7, and 8). An adaptation process permits further [^{3}H]cAMP secretion to be elicited without need of a recovery period when the external cAMP concentration is increased (Fig. 8). This formulation also explains why the magnitude of each serial response is influenced by the previous stimulus concentration, since amoebae would continuously monitor environmental cAMP levels.

Two contrasting features of the cAMP relay process are revealed by responses to multistep stimuli. The total magnitude of responses to a multistep stimulus between C_1 and C_2 is approximately equal to the single response to a direct C_1 to C_2 jump. The amount of [^{3}H]cAMP released is independent of the number, magnitude, and duration of the steps on the path from C_1 to C_2 (Table I). On the other hand, the sum of the durations of the individual responses does depend on the number and duration of the steps on this path, i.e., increasing cAMP in multiple steps greatly extends the total response time (Figs. 8 and 9). Thus, in cAMP relay of *D. discoideum*, magnitudes of responses are additive while response durations are not. (Bacterial chemotaxis also involves a process of adaptation; however, it appears, in that case, that the durations of responses are additive [1, 25].)

All of our data on the relay response and its termination suggest that amoebae continuously monitor extracellular cAMP stimuli by the fractional occupancy of a finite number of cAMP relay receptors. (*a*) The continuous presence of cAMP is necessary to maintain a relay response (Figs. 1 and 2). (*b*) Responses are a function of the magnitude of the stimulating concentration step and show saturation at high doses (Fig. 6). (*c*) The extent of adaptation closely reflects the external cAMP concentration, since adaptation can be repeatedly overcome by twofold increments in the stimulus (Fig. 9). (*d*) Although relay receptors may represent only a subset of the total [^{3}H]cAMP binding sites, our dose-response curve resembles the binding curve for cAMP (reference 9 and our own unpublished binding data for NC-4).

As a working hypothesis, which embraces all of our data, we propose that cAMP-induced cAMP secretion in *D. discoideum* involves two antagonistic cellular processes which control the generation and termination of the response (1, 13). In this scheme, an increase in the fractional occupancy of cAMP relay receptors would lead to increases, at different rates, in the levels of two control parameters—S (signal) and A (for adaptation). Both S and A would approach new values specified by relay receptor occupancy, with A reaching this value after S. While S exceeded A, cAMP accumulation and secretion would proceed. When the value of A matched S, full adaptation would prevail. A fresh cAMP increment could reinitiate the cycle, at any time, raising both S and A to still higher values. Reducing receptor occupancy would reduce S and A and increase cellular sensitivity to subsequent stimuli.

Finally, the process of adaptation described in

this report accounts for the apparent refractory period which organizes cyclic AMP signal propagation through fields of aggregating amoebae.

The authors thank Mr. K. Tomchik for his expert assistance, Dr. R. Clark and Ms. M. Dinauer for helpful discussions, Dr. R. Haselkorn and Ms. K. Hoffmann for critical review of the manuscript, and Ms. S. Parks for typing.

This research was supported by US Public Health Service Grant GM 22321. P. N. Devreotes is a postdoctoral Fellow of the Damon Runyon-Walter Winchell Cancer Fund DRG (178F). T. L. Steck is the recipient of a Faculty Research Award from the American Cancer Society.

Received for publication 5 June 1978, and in revised form 15 September 1978.

REFERENCES

1. BERG, H., and P. TEDESCO. 1975. Transient response to chemotactic stimuli in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **72**:3235–3239.
2. BONNER, J. T. 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. *J. Exp. Zool.* **106**:1–26.
3. COHEN, M., and A. ROBERTSON. 1971. Wave propagation in the early stages of aggregation of cellular slime molds. *J. Theor. Biol.* **31**:101–118.
4. DEVREOTES, P., P. DERSTINE, and T. STECK. 1978. Cyclic 3′,5′ AMP relay in *Dictyostelium discoideum*. I. A technique to monitor responses to controlled stimuli. *J. Cell Biol.* **80**:291–299.
5. GERISCH, G., and D. MALCHOW. 1976. Cyclic AMP receptors and the control of cell aggregation in *Dictyostelium*. *Adv. Cyclic Nucleotide Res.* **7**:49–68.
6. GERISCH, G., and U. WICK. 1975. Intracellular oscillations and release of cyclic AMP from *Dictyostelium* cells. *Biochem. Biophys. Res. Comm.* **65**:364–370.
7. GERISCH, G., Y. MAEDA, D. MALCHOW, W. ROOS, U. WICK, and B. WEBSTER. 1977. Cyclic AMP signals and the control of cell aggregation in *Dictyostelium discoideum*. *In* Development and Differentiation in the Cellular Slime Moulds. Cappuccinelli and Ashworth, editors. Elsevier North-Holland Inc., New York.
8. GOLDBETER, A., and L. SEGAL. 1977. Unified mechanism for relay and oscillation of cyclic AMP in *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. U. S. A.* **74**:1543–1547.
9. GREEN, A., and P. C. NEWELL. 1975. Evidence for the existence of two types of cAMP binding sites in aggregating cells of *Dictyostelium discoideum*. *Cell.* **6**:129–136.
10. KAHN, R. 1976. Membrane receptors for hormones and neurotransmitters. *J. Cell Biol.* **70**:261–286.
11. KING, C., and W. FRAZIER. 1977. Reciprocal periodicity in cyclic AMP binding and phosphorylation of differentiating *Dictyostelium discoideum* cells. *Biochem. Biophys. Res. Comm.* **78**:1093–1099.
12. KLEIN, C., and M. JULIAN. 1977. cAMP induced changes in cAMP binding sites on *Dictyostelium discoideum* amoebae. *Cell.* **10**:329–335.
13. MACNAB, R. M., and D. E. KOSHLAND, JR. 1972. The gradient-sensing mechanism in bacterial chemotaxis. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2509–2512.
14. MALCHOW, D., and G. GERISCH. 1974. Short term binding and hydrolysis of cAMP by aggregating *Dictyostelium* cells. *Proc. Natl. Acad. Sci. U. S. A.* **71**:2423–2427.
15. NEWELL, P. 1977. Aggregation and cell surface receptors in cellular slime molds. *In* Microbial Interactions (Receptors and Recognition, Series B, Vol. 3). J. L. Reissig, editor. Chapman & Hall, Ltd., London.
16. RANG, H., and J. M. RITTER. 1970. On the mechanism of desensitization of cholinergic receptors. *Mol. Pharmacol.* **6**:357–382.
17. ROBERTSON, A., D. DRAGE, and M. COHEN. 1972. Control of aggregation in *Dictyostelium discoideum* by an external periodic pulse of cyclic adenosine monophosphate. *Science (Wash. D. C.).* **175**:333–335.
18. ROOS, W., and G. GERISCH. 1976. Receptor-mediated adenylate cyclase activation in *Dictyostelium discoideum*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **68**:170–172.
19. ROOS, W., V. NANJUNDIAH, D. MALCHOW, and G. GERISCH. 1975. Amplification of cyclic AMP signals in aggregation cells of *Dictyostelium discoideum*. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **53**:139–142.
20. ROOS, W., C. SCHEIDEGGER, and G. GERISCH. 1977. Adenylate cyclase activity oscillations as signals for cell aggregation in *Dictyostelium discoideum*. *Nature (Lond.).* **266**:259.
21. SHAFFER, B. M. 1957. Aspects of aggregation in cellular slime molds. *Am. Nat.* **91**:19–35.
22. SHAFFER, B. M. 1958. Integration in aggregating cellular slime molds. *Q. J. Microsc. Sci.* **99**:103–121.
23. SHAFFER, B. M. 1962. The acrasina. *Adv. Morphog.* **2**:109–182.
24. SHAFFER, B. M. 1975. Secretion of cyclic AMP induced by cyclic AMP in the cellular slime mold *Dictyostelium discoideum*. *Nature (Lond.).* **255**:549–552.
25. SPUDICH, J. L., and D. E. KOSHLAND, JR. 1975. Quantitation of the sensory response in bacterial chemotaxis. *Proc. Natl. Acad. Sci. U. S. A.* **72**:710–713.