Cyclic 3′,5′-AMP Relay in *Dictyostelium discoideum* III. The Relationship of cAMP Synthesis and Secretion during the cAMP Signaling Response

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ABSTRACT Refinement of a perfusion technique permitted the simultaneous measurement of cAMP-elicited [^{3}H]cAMP secretion and intracellular [^{3}H]cAMP levels in sensitive *D. discoideum* amoebae. These data were compared with measurements of the rate of [^{32}P]cAMP synthesis by extracts of amoebae sonicated at different times during the cAMP signaling response. cAMP stimulation of intact cells led to a transient activation of adenylate cyclase, which was blocked if 10^{-4} M NaN_3 was added with the stimulus. During responses elicited by 10^{-6} M cAMP, 10^{-8} M cAMP, and an increment in cAMP from 10^{-8} M to 10^{-7} M, the rate of cAMP secretion was proportional to the intracellular cAMP concentration. Removal of a 10^{-6} M cAMP stimulus 2 min after the initiation of the response led to a precipitous decline in intracellular cAMP. This decline was more rapid than could be accounted for by secretion alone, suggesting intracellular phosphodiesterase destruction of newly synthesized cAMP. Employing these data and a simple rate equation, estimates of the time-course of the transient activation of adenylate cyclase and the rate constants for cAMP secretion and intracellular phosphodiesterase activity were obtained. The calculated rate of cAMP synthesis rose for ~1 to 2 min, peaked, and declined to approach prestimulus levels after 3 to 4 min. This time-course agreed qualitatively with direct measurements of the time-course of activation, indicating that the activation of adenylate cyclase is a major element in determining the time-course of the cAMP secretion response.

Dictyostelium discoideum grows as free-living amoebae, feeding upon bacteria until the food supply is exhausted or removed. The amoebae then aggregate to assemble multicellular structures, each containing ~10^5 cells. Through a process of morphogenesis and differentiation, fruiting bodies are formed, consisting of a ball of spores held aloft on a slender cellular stalk. Aggregation is guided by chemotaxis toward cyclic adenosine 3′,5′-monophosphate (cAMP) signals. Because amoebae also respond to cAMP signals by secreting additional cAMP, the signal is relayed from cell to cell over long distances (up to 10 mm). Waves of cAMP, which emanate from centers with a regular periodicity of 5–10 min, can be visualized in monolayers of aggregating amoebae.[1] After the passage of each wave, extracellular cAMP is destroyed by membrane-bound and secreted phosphodiesterases. The result of these processes is the orderly assembly of a multicellular form (1, 6, 15, 17).

Our goal is to understand the mechanism of cAMP signaling. This process apparently involves the sequential binding of the signal molecule to the cell surface receptors for cAMP, transduction of the event to the cytoplasmic side of the membrane, activation of adenylate cyclase, accumulation of intracellular cAMP, and, finally, its secretion into the medium. No systematic and quantitative kinetic analysis of this sequence of events has been published. Furthermore, previous studies have employed cell suspensions in which the level of the stimulus and the recovery of the secreted response were not controlled (7, 8). We have therefore measured the time-course of activation of adenylate cyclase in response to extracellular cAMP. We also followed the kinetics of accumulation of intracellular cAMP in response to exogenous cAMP signals and the concomitant release of this nucleotide into the medium, using a filter-perfusion apparatus. These data were correlated to assess the role of adenylate cyclase activity, intracellular phosphodiesterase activity, and the secretion of cAMP in the regulation of the signaling response.

[1] Tomchik, K. J., and P. N. Devreotes. Manuscript in preparation.

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MATERIALS AND METHODS

Growth and Development of Ax-3

Ax-3, the axenic strain of *D. discoideum* (14, 22), was obtained from W. C. Summers of Yale University. Ax-3 amoebae were grown in suspension at 22°C in HL-5 medium (per liter: 10 g of dextrose, 10 g of protease peptone no. 3 [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract [Difco], 0.965 g of $Na_2H_2PO_4 \cdot 7\ H_2O$, 0.486 g of KH_2PO_4, 100,000 U of penicillin, and 0.1 g of streptomycin [14]). Cultures were started from frozen spore stocks and maintained for no more than 3 mo. Doubling times ranged from 8 to 12 h. Cells were passed before the culture had reached a density of 5×10^6 cells/ml. Cell density in the stationary phase was 2×10^7 cells/ml. To initiate differentiation by starvation, cells at a density of $2–5 \times 10^6$ cells/ml were washed by two centrifugations (600 *g* for 3 min) at 4°C in M-KK_2 buffer (2.31 g of KH_2PO_4, 1.00 g of K_2HPO_4, 0.5 g of $MgSO_4$ per liter solution, pH 6.2), resuspended in M-KK_2 at a density of 2×10^7 cells/ml, and shaken for 5–6 h (gyratory shaker, model G-2, 1-cm radius, 225 rpm; New Brunswick Scientific Co., Edison, N. J.) at 22°C.

cAMP and Adenylate Cyclase Activity in Ax-3 Suspension

After 5–6 h of starvation in suspension, cells were washed twice in M-KK_2 and resuspended at a density of 2×10^7 cells/ml. After ~20 min at 22°C, the cAMP signaling responses of the cells were synchronized in suspension by adding 5 μl of 10^{-6} M cAMP per milliliter of cell suspension every 7 min. The final stimulus was usually 10 μl 10^{-4} M cAMP, 1 M dithiothreitol (DTT) per milliliter of suspension. The concentration of total cAMP (intracellular plus extracellular cAMP) in the cell suspensions was estimated with the erythrocyte ghost isotope dilution assay as previously described (3). Adenylate cyclase activity measurements followed the methods of Roos and Gerisch (19). 500 μl of cell suspension were mixed with 50 μl of 2 M sucrose, 10 mM EDTA, 0.5 M Tris-HCl (pH 8) and 100 mM DTT and sonicated for 2–5 s (on a Branson Sonifier, model W-140, setting 3.5; Branson Sonic Power Co., Danbury, Conn.). 100 μl of the sonicate were added to 10 μl of 50 mM $MgCl_2$, 100 mM DTT, 50 mM cAMP, 1 M creatine phosphate, 1 mg/ml creatine phosphokinase (Sigma Chemical Co., St. Louis, Mo.), 5 mM ATP, and 250 μCi/ml ATP-(γ-^{32}P). After 1 min at 22°C, 100 μl of 1% SDS was added. [^{32}P]cAMP was purified according to the methods of Salomon et al. (21).

Growth of NC-4

Dictyostelium discoideum Raper, strain NC-4, and *Enterobacter aerogenes* (stock nos. 11735 and 1304B, respectively) were obtained from the American Type Culture Collection.

Liquid *E. aerogenes* cultures were prepared by growing a colony of *E. aerogenes* in 50 ml of nutrient broth (5.0 g of dextrose, 5.0 g of bactopeptone [Difco], 0.5 g of yeast extract [Difco] per liter of M-KK_2) for 1–2 days without shaking. *E. aerogenes* was propagated as colonies by streaking a liquid culture onto nutrient agar plates (2% agar in nutrient broth). After 2 d of growth at room temperature, plates were stored at 4°C for up to a month before use.

To grow and propagate NC-4 cells, spores were harvested from fruiting bodies and mixed with a 1- to 2-day liquid *E. aerogenes* culture. After spore density was determined with a hemocytometer, aliquots were mixed with 0.2 ml of liquid *E. aerogenes* culture and spread evenly on nutrient agar plates. Plating densities were calculated from the estimated doubling time of 4 h to yield 5×10^7 NC-4 cells/plate after a 24-h incubation at 22°C. After growth to higher densities, patchy clearing of the confluent bacterial lawn occurs. Plates were harvested for starvation after 20–24 h of growth, with some reserved for development to fruiting bodies. Every 3–4 mo, NC-4 cultures were restarted from frozen spore stocks. Spores were plated as described and passed through the entire life-cycle two to three times before being used to grow amoebae for experiments.

[^{3}H]Adenosine Labeling and Conditions for Development of NC-4

NC-4 amoebae were washed off nutrient agar plates with M-KK_2 and freed of bacteria by two to three centrifugations (600 *g* for 3 min) at 4°C. Amoebae were then shaken at a density of 5×10^6 cells/ml with [^{3}H]adenosine-labeled *E. coli* (3). After 3 h at 22°C, amoebae were freed of labeled bacteria by two centrifugations (600 *g* for 3 min). Labeled amoebae were resuspended in M-KK_2 and transferred to nutrient-free agar plates (2% agar in M-KK_2) in a volume of 200 μl (for petri dishes of 35-mm diameter, Falcon 1008; Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) or 400 μl (60-mm diameter petri dishes, Falcon 1007). Plating density was $0.7–1 \times 10^6$ cells/cm^2. Cells were spread by shaking and swirling the plates. Lids were not put on until excess surface moisture had disappeared after 10–20 min. Plates were then incubated in a humidified chamber in the dark at 7°C for 17–18 h or at 22°C for 5–7 h. Before using amoebae developed at 7°C in an experiment, the plate was transferred to a 22°C incubator for 30–45 min. When the first signs of aggregation became visible, cells were harvested by gently flushing the plates with 1–2 ml of M-KK_2. Identical aliquots were pipetted onto eight filters (Millipore SSWPO 1300, Millipore Corp., Bedford, Mass.) in the perfusion apparatus, as described below. All experiments were carried out at 19°–22°C. The number of cells per filter ranged from $0.5–1.5 \times 10^6$ cells. Total filter radioactivity, determined by counting an aliquot of amoebae in 1 ml 1% Triton and 7 ml Triton-toluene fluor (3), ranged from $0.5–3 \times 10^6$ cpm.

Measurement of Intracellular and Extracellular cAMP

PERFUSION APPARATUS: Our objective was to measure changes in intracellular and secreted cAMP elicited by exogenous cAMP stimuli of defined magnitude and duration. *D. discoideum* amoebae can modify the extracellular cAMP concentration in their environment both by secretion of additional cAMP and by phosphodiesterase destruction of cAMP. However, we have shown that rapid perfusion maintains a constant exogenous cAMP concentration and removes secreted cAMP effectively (3). Equilibrium binding of cAMP to surface receptors is rapidly achieved (11). Therefore, during each successive drop (5 s), the receptor occupancy should become adjusted to the applied cAMP concentration and little secreted cAMP should remain bound to cellular sites. Because the perfusion technique also rapidly separates secreted cAMP from cells, it permits the simultaneous estimation of intracellular and secreted cAMP.

The eight-filter perfusion apparatus employed is illustrated in Fig. 1. The details of its construction (i.e., filter holders, tubing connections, etc.) follow those described for a single filter perfusion apparatus (3). Perfusion solutions were delivered via an eight-channel Gilson Minipuls (Gilson Medical Electronics, Inc., Middleton, Wis.) pump. Flow rates were made nearly identical among the channels at 1 drop/5–6 s. Perfusion solutions were abruptly switched as follows. The lines leading to the pump were transferred to the new solution. This introduced an air bubble into each of the pump lines. Just before the air bubble emerged, the bar supporting the pump lines was lifted from above the filters. The "rabbit" control of the Gilson Minipuls pump was used to rapidly clear the air bubble from the lines, starting the flow of the new solution. The support bar was then repositioned above the filters. The entire procedure required ~15 s, during which time the filters were not perfused. Time 0 was taken as the moment the first drop of new solution fell onto the filters.

SYNCHRONIZATION OF CELLS: At the time of harvest, the [^{3}H]adenosine-labeled amoebae showed visible signs of aggregation. Because some of the aggregating cells are likely to be signaling when harvested, they initially might vary in their ability to respond to external cAMP. To synchronize the amoebae, freshly harvested amoebae were loaded onto filters and perfused with M-KK_2 for 3–5 min. A stimulus of 10^{-8} M cAMP was applied for 2–3 min and the perfusion buffer was then switched back to M-KK_2 alone. After 8–10 min of recovery, the test stimulus was given, as indicated in Results, and intracellular and extracellular [^{3}H]cAMP were monitored.

PURIFICATION OF CAMP: To quantitatively compare amounts of intracel-

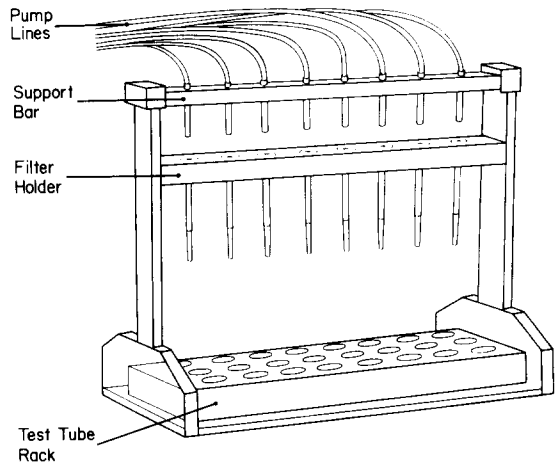

FIGURE 1 An eight-filter perfusion apparatus. Drawn to the scale of a filter holder, which is 13 mm in diameter.

lular and secreted [^{3}H]cAMP, [8-^{14}C]cAMP (53.1 mCi/mmol, New England Nuclear, Boston, Mass.) was used as an internal standard. Secreted [^{3}H]cAMP was collected into 100 μl of a phosphodiesterase stopping solution (8% formic acid, 10^{-3} M cAMP) containing 50–80 cpm of [^{14}C]cAMP. At each time-point, one filter was removed from the apparatus and inverted into 250 μl of stopping solution containing the same number of counts per minute as the 100 μl of extracellular stopping solution. After purification, the [^{3}H]cAMP/[^{14}C]cAMP ratio for the filter was used to quantitate the amount of [^{3}H]cAMP associated with the cells at a time-point, while the [^{3}H]cAMP/[^{14}C]cAMP ratio for the perfusate gave the total amount of [^{3}H]cAMP secreted up to that moment.

The extracellular perfusates (usually 3–5 ml) and 200-μl aliquots of the material extracted from the filters were frozen at −20°C. After drying under vacuum, the residue was resuspended in 1 ml of M-KK$_2$ and applied to a 1-ml Bio-Rad AG 50W-X4 column (Bio-Rad Laboratories, Richmond, Calif.). Columns were eluted as described previously (3) except that the eluate containing cAMP was eluted directly into a second column containing 1 g of neutral alumina (Bio-Rad) preequilibrated with 50 mM Tris-HCl, pH 7.5. The alumina columns were eluted directly into scintillation vials with 6 ml of 100 mM Tris-HCl (pH 7.5). The solution in the vials was then evaporated to dryness at 65°C, and samples were processed and counted as previously described (3). Recovery of [^{3}H]cAMP was ~60% for both filter extracts and extracellular perfusates.

The effectiveness of the purification method for intracellular cAMP was tested by analysis of the radioactivity eluted from the alumina columns. In this experiment, alumina columns were eluted with 100 mM *N*-ethyl morpholine (pH 7.5), which elutes an identical amount of radioactivity as 100 mM Tris-Cl (pH 7.5). Samples were evaporated to dryness, resuspended in 100 μl H_2O, and examined by thin-layer chromatography as previously described (3). Little of the cell-associated radioactivity present before application of the stimulus was [^{3}H]cAMP, whereas essentially all of the increase in cell-associated radioactivity after stimulation was [^{3}H]cAMP. The fold increase in cell-associated [^{3}H]cAMP is therefore greater than appears in the experiments reported here. This control experiment, however, suggests that a low, constant background can be subtracted from each time-point and that changes in the ^{3}H/^{14}C ratio accurately reflect changes in the level of intracellular [^{3}H]cAMP.

Quantitative Analysis of Intracellular and Extracellular cAMP

To compare intracellular levels of cAMP and cAMP secretion rate, it was necessary to compute the secretion rate from the graph of extracellular [^{3}H]-cAMP/[^{14}C]cAMP ratios vs. time. This was accomplished by first hand-fitting a curve to the extracellular cAMP data points. This curve was then entered into a specially designed computer system (18) using a sonic digitizing pen (Graph/Pen, Scientific Accessories Corp., Southport, Conn.). The derivative of this curve was computed using Lagrangian interpolation polynomials (10). Small irregularities in the curve were introduced because the curve was traced by hand to enter the data into the computer system. Median smoothing of the derivative was used to eliminate single anomalous points; however, some small fluctuations in the computed derivative remained (e.g., Fig. 2) to which no significance is attached. The same procedure was followed for intracellular [^{3}H]cAMP/[^{14}C]cAMP ratios to compute dI/dt (I, intracellular cAMP level; t, time) for purposes of computing adenylate cyclase activity.

RESULTS

Responses Elicited by Continuous Stimuli of High Concentration

Our initial objective was to correlate intracellular cAMP levels with the rate of secretion of cAMP during responses elicited by a nearly saturating stimulus, 10^{-6} M cAMP. As previously demonstrated (3, 4), when the stimulus concentration was held constant by rapid perfusion with 10^{-6} M cAMP, the cAMP secretion rate rose and fell with a characteristic time-course (Fig. 2, dotted curve). The cAMP secretion rate was calculated by taking the derivative of the curve showing the total cAMP secreted as a function of the stimulus duration (Fig. 2, open symbols). The rate rose geometrically for 2–3 min and returned to baseline levels within 10–15 min. The decline in the cAMP secretion rate began abruptly and had rapid ($t_{1/2}$ = 2 min) and slow ($t_{1/2}$ = 5–6 min) components. Thus, the major portion of the profile had a "sawtooth" appearance

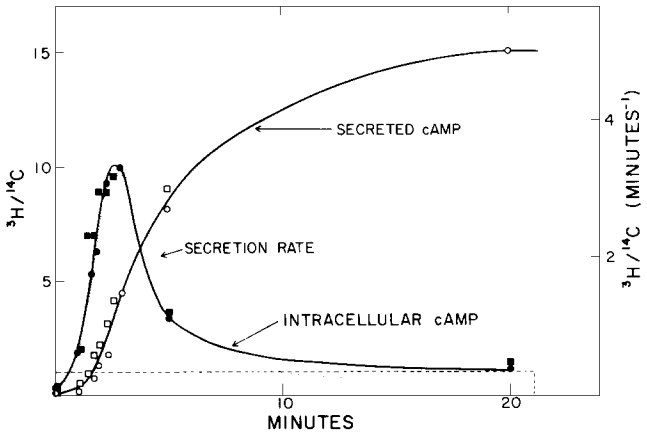

FIGURE 2 Response to a continuous 10^{-6} M cAMP stimulus. Labeled, differentiated NC-4 amoebae were placed in the perfusion apparatus and synchronized with cAMP as described in Materials and Methods. The perfusion solution was then switched to one containing 10^{-6} M cAMP (time 0, above). This stimulus was maintained for 20 min (dashed lines). Plotted is the ratio of [^{3}H]cAMP/[^{14}C]cAMP recovered in each of the intracellular (closed symbols) or extracellular (open symbols) samples. The data from two experiments carried out on the same day are shown (squares or circles). Amoebae from one nutrient-free agar plate were used for each experiment. The data from the second experiment (circles) were normalized such that the final extracellular [^{3}H]cAMP/[^{14}C]cAMP ratio equaled that of the first experiment (squares). The unnormalized final extracellular ratio in the second experiment was 13.2. The thin dotted line is the cAMP secretion rate calculated as the derivative of the accumulated extracellular [^{3}H]cAMP/[^{14}C]cAMP, scaled so that its peak height was equal to that of the intracellular ^{3}H/^{14}C ratio. The experiment was repeated four times with similar results.

followed by a slow decline to prestimulus levels. Perfusion with higher stimulus concentration (10^{-4} M) elicited responses of similar magnitude and time-course.

When cells were treated with a sustained stimulus of 10^{-6} M cAMP, the level of intracellular cAMP rose rapidly from basal levels and then fell spontaneously (Fig. 2). The falling phase of the intracellular cAMP level appeared to have two components. Intracellular cAMP dropped to ~30% of its peak value after 5 min of stimulation. It then declined slowly and by 20 min, the longest time monitored, it had not quite reached the prestimulus level. The change in intracellular cAMP levels was thus qualitatively similar to that in the cAMP secretion rate. They rose with the same time-course, peaked after about 2 min, and declined at nearly the same rate.

Responses Elicited by Continuous Stimuli of Low Concentration

Fig. 3 shows a representative response to a continuous stimulus of 10^{-8} M cAMP. (The total amount of cAMP secreted at this stimulus concentration is ~10–30% of that secreted in response to 10^{-6} M cAMP.) The time-course of the response was similar to that elicited by 10^{-6} M cAMP. The changes in both intracellular cAMP levels and cAMP secretion rates had a sawtooth profile; however, the half-width was slightly narrower in response to 10^{-8} M cAMP than to 10^{-6} M cAMP. As with responses to 10^{-6} M cAMP, the time-course of the intracellular cAMP accumulation and cAMP secretion rate were quite similar. Thus, the secretion rate was directly proportional to the intracellular cAMP level during the response to both subsaturating and saturating stimuli.

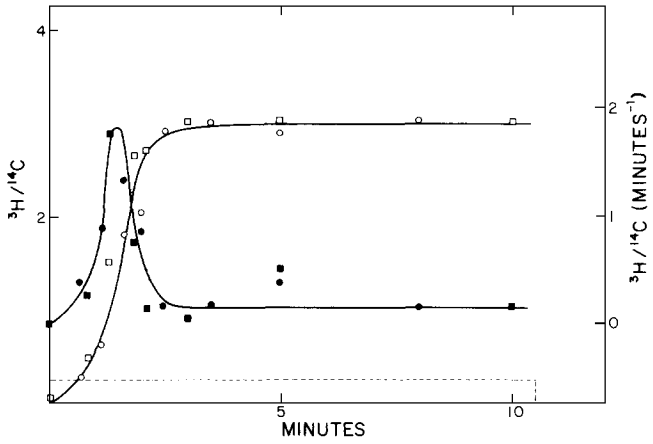

FIGURE 3 Response to a continuous 10^{-8} M cAMP stimulus. Protocal was identical to that in Fig. 2 except that a stimulus of 10^{-8} M cAMP was given at time 0. This stimulus was maintained for 15 min (as indicated by the dashed lines). The [^{3}H]cAMP/[^{14}C]cAMP ratio is plotted for each of the intracellular (closed symobls) and extracellular (open symbols) samples. The unnormalized final extracellular ratio in the second experiment (circles) was 3.12. Thin dotted line is the calculated secretion rate. The experiment was repeated three times with similar results.

Responses Elicited by Increments in Stimulus Concentration

The response to a given cAMP stimulus eventually subsides because of adaptation, but an increment in the stimulus concentration will elicit a second response (4). The time-course of the response to the increment is similar to that elicited by the initial stimulus except that the initial rate of increase in cAMP secretion rate is more rapid and the peak secretion rate occurs sooner (5).

In Fig. 4, we demonstrate that the more rapid increase in the cAMP secretion rate after an increment in the stimulus reflects an increased rate of accumulation of intracellular cAMP. A 5-min stimulus of 10^{-8} M cAMP was immediately followed by one of 10^{-7} M cAMP. The changes in intracellular and extracellular cAMP were monitored during the second response. The intracellular cAMP level rose more rapidly in response to the increment than in response to the initial stimulus (cf. Fig. 3). Intracellular cAMP increased from near basal levels at the onset of the 10^{-7} M cAMP stimulus, reached a peak value by 30 s, and then rapidly declined. The accelerated rise in intracellular cAMP shown in Fig. 4 accounts for the rapid rise in the secretion rates typically observed in response to increments in the cAMP stimulus (5).

Time-course of Adenylate Cyclase Activity during Continuous Stimulation

It has not been possible to activate *D. discoideum* adenylate cyclase with cAMP in cell homogenates. When cell suspensions were stimulated with cAMP before lysis, the enzyme in the homogenate showed increased activity that persisted for ~45 s (7, 12, 19, 20). Homogenates prepared several minutes after the addition of the stimulus no longer showed increased adenylate cyclase activity. It is unclear whether the transient elevation of enzyme activity reflected a spontaneous deactivation of the enzyme or the destruction of the extracellular cAMP stimulus.

To test the effects of prolonged cAMP stimulation on adenylate cyclase activity, we sought a method to maintain a relatively constant cAMP stimulus concentration. We stimulated suspensions of amoebae with 10^{-6} M cAMP and added 10 mM DTT, a phosphodiesterase inhibitor (2, 9), to maintain the stimulus at a high level. As seen in Fig. 5, adenylate cyclase activity progressively increased in response to the stimulus, reaching a maximum after ~1 min of stimulation. Despite the persistence of the stimulus, adenylate cyclase activity then rapidly declined, approaching prestimulus rates within 3 min of the onset of the stimulus. The slight elevation persisting at subsequent times may be the source of the slow decline in the intracellular cAMP level during a continuous 10^{-6} M stimulus in Fig. 2.

Effect of Sudden Removal of a cAMP Stimulus

We previously demonstrated that the removal of the stimulus at any time during a response resulted in a rapid decline in the cAMP secretion rate (3, 4). This observation is consistent with the rapid dissociation of cAMP-receptor complexes ($t_{1/2}$ = 4 s) (16). The abrupt decrease in the secretion rate suggests that continuous occupation of surface cAMP binding sites is necessary to maintain either cAMP transport or the continued production of intracellular cAMP. To evaluate this issue, cells were perfused for 2 min with 10^{-6} M cAMP and then with buffer alone. Intracellular cAMP levels began to fall as soon as the stimulus was removed (Fig. 6). Secretion of cAMP continued until intracellular cAMP had declined to basal levels. This result indicates that cAMP production depends on the continued presence of the stimulus, whereas secretion of cAMP does not.

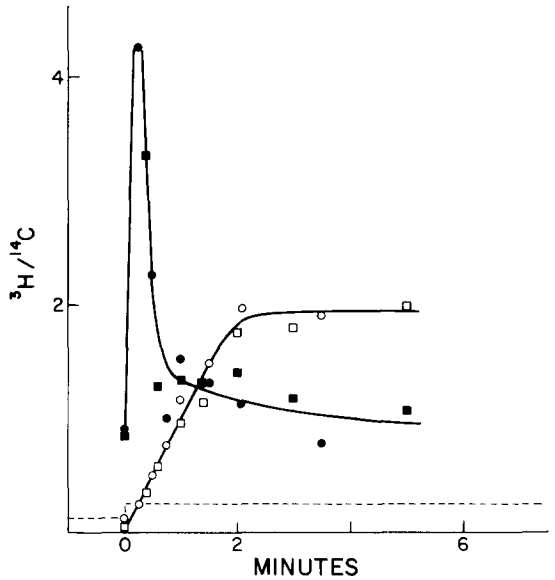

FIGURE 4 Responses to a stimulus increment from 10^{-8} M to 10^{-7} M cAMP. Protocol was similar to that in Fig. 2. However, after a stimulus of 10^{-8} M cAMP had been applied for 5 min, the concentration of the cAMP stimulus was then suddenly increased to 10^{-7} M (time 0, above). The [^{3}H]cAMP/[^{14}C]cAMP ratio is plotted for each of the intracellular (closed symbols) and extracellular (open symbols) samples. Again, the results of two experiments are shown. The unnormalized final extracellular ratio in the second experiment (circles) was 3.8. Results similar to those shown in Fig. 4 were also obtained when the external cAMP concentration was raised to 5 × 10^{-8} M cAMP immediately after a 5- or 7- min stimulus of 10^{-8} M cAMP.

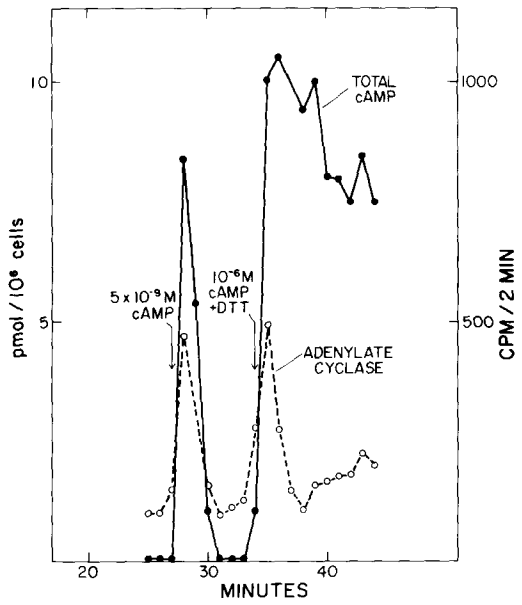

FIGURE 5 Time-course of adenylate cyclase activity during continuous cAMP stimulation. Unlabeled Ax-3 amoebae in suspension were synchronized as described in Materials and Methods. The experiment illustrates the fourth and fifth synchronization cycles (28 and 35 min). At 34 min, the suspension was adjusted to 10^{-6} M cAMP, 10 mM DTT as indicated. At 1-min intervals, 50 μl of suspension were removed for determination of total cAMP; 500 μl were removed, sonicated, and 100 μl used for determination of adenylate cyclase activity. 1,000 cpm of [^{32}P]cAMP corresponds to ~25 pmol of cAMP. The experiment was repeated four times with similar results.

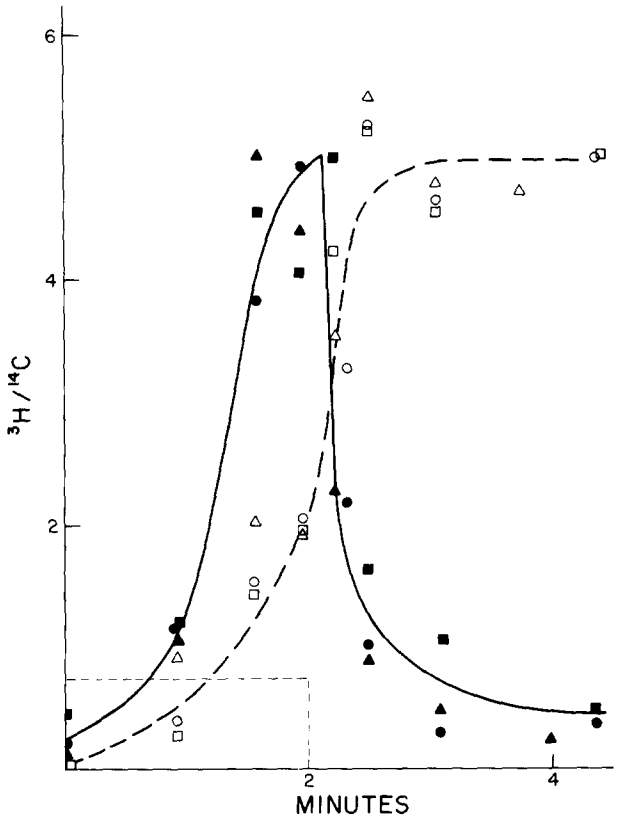

FIGURE 6 Response to a 2-min 10^{-6} M cAMP stimulus. Protocol was similar to that in Figs. 2 and 3. A 10^{-6} M cAMP stimulus was applied (time 0, above) for 2 min, as indicated by the dashed lines, and followed by buffer alone. Plotted is the ratio of [^{3}H]cAMP/[^{14}C]cAMP recovered in each of the intracellular (closed symbols) or extracellular (open symbols) samples. The data from three experiments carried out on the same day are shown. All of the data from the second and third experiments (circles and triangles) were normalized such that the final extracellular [^{3}H]cAMP/[^{14}C]cAMP ratio equaled that of the first experiment (squares). The actual final extracellular ratio in the second experiment (circles) was 5.8 and that of the third (triangles) was 4.7. The experiment was repeated twice with similar results.

Inhibition of Adenylate Cyclase Activation by NaN_3

The signaling response is rapidly inhibited by NaN_3 (Fig. 7). When 10^{-4} M NaN_3 was added with a 10^{-6} M cAMP stimulus, no cAMP secretion was elicited. When 10^{-4} M NaN_3 was introduced after 75 s of stimulation with 10^{-6} M cAMP, the secretion of cAMP declined rapidly. The fall in the secretion rate was essentially identical to that observed when the stimulus itself was removed.

NaN_3 does not antagonize cAMP binding to surface sites, in that the equilibrium binding of 10^{-8} M or 5×10^{-7} M [^{3}H]-cAMP to cells was unaffected by 10^{-4} M NaN_3 (data not shown). Furthermore, the inhibition of the signaling response is not attributable solely to the depletion of ATP as a substrate for the adenylate cyclase. NaN_3, an inhibitor of oxidative phosphorylation, does cause a rapid reduction in ATP levels in

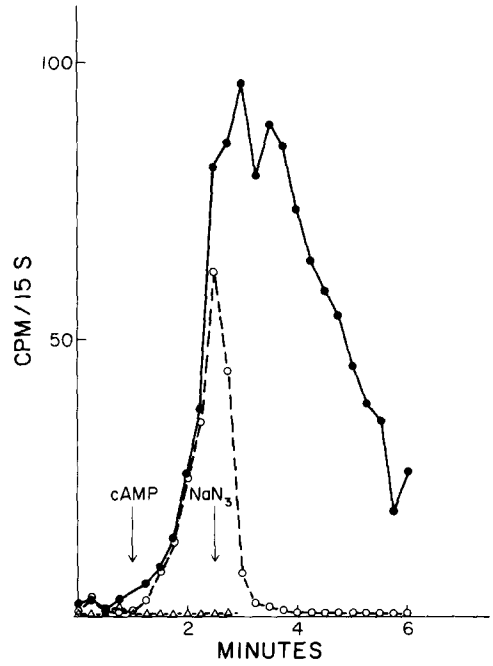

FIGURE 7 Inhibition of responses to cAMP by NaN_3. In this experiment, [^{3}H]adenosine-labeled, aggregating NC-4 amoebae from a single nutrient-free agar plate were divided among three separate filters. The four-filter perfusion apparatus employed is similar to that described in detail in Fig. 3. After transfer to the filters, amoebae were perfused for 20 min with M-KK_2, stimulated with 10^{-8} M cAMP for 2 min, and perfused with M-KK_2 for 15 min. Stimuli were then introduced simultaneously, as indicated by the first arrow. One filter was perfused with a continuous 10^{-6} M cAMP stimulus (—●—). The second filter was perfused with a combination of 10^{-6} M cAMP and 10^{-4} M NaN_3 (—△—). The third filter received 10^{-6} M cAMP alone for 75 s, followed by 10^{-6} M cAMP plus 10^{-4} M NaN_3, as indicated by the second arrow (- -○- -). The experiment was repeated five times with similar results.

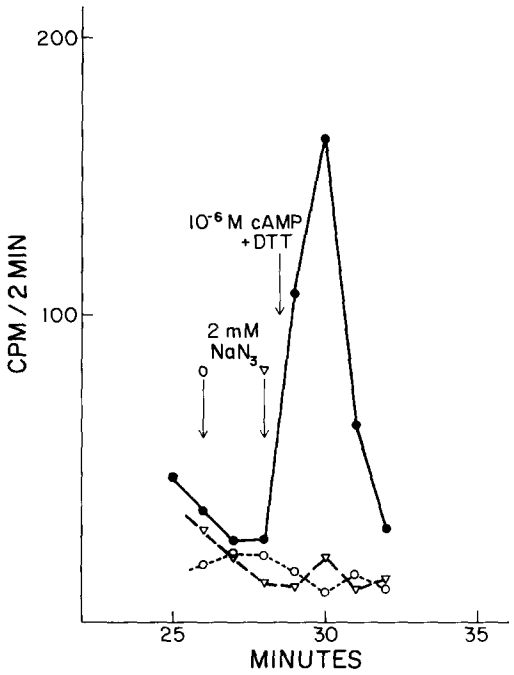

FIGURE 8 Inhibition of activation of adenylate cyclase by NaN_3. 10 ml of a suspension of unlabeled Ax-3 amoebae were synchronized as described in Materials and Methods. The experiment illustrates the fourth synchronization cycle (28 min). At 26 min (- -○- -) and 28 min (—▽—), 3 ml were removed to separate 10-ml beakers and adjusted to 10^{-4} M NaN_3. The remaining 4 ml served as control (—●—). At 28.5 min, all three cell suspensions were adjusted to 10^{-6} M cAMP, 10 mM DTT. Each minute, 500-μl aliquots of each suspension were removed and sonicated, and 100 μl of each were processed in parallel for adenylate cyclase activity. The experiment was repeated three times with similar results.

these cells ($t_{1/2}$ = 8 s).[2] However, as illustrated in Fig. 8, NaN_3 had a direct effect on the activation of adenylate cyclase. In this experiment, 10^{-4} M NaN_3 was added to a cell suspension just before the introduction of a cAMP stimulus. The activity of adenylate cyclase measured in sonicates did not increase, even though 0.5 mM ATP was present in the reaction mixture. NaN_3 added to sonicates did not inhibit activated adenylate cyclase. These results, taken together, suggest that there is a step between the binding of cAMP to surface sites and the activation of adenylate cyclase that can be blocked by NaN_3.

DISCUSSION

It is evident that the rate of cAMP secretion is closely linked to the level of intracellular cAMP under a variety of conditions. The cAMP secretion rate rose and fell in parallel with intracellular cAMP in response to sustained stimuli (Figs. 2–4). Upon removal of the stimulus, both intracellular cAMP and the cAMP secretion rate declined abruptly (Fig. 6). There is thus no evidence for regulation of the response at the cAMP secretion step. cAMP is synthesized and transiently accumulated before release, with no long-term storage. Under a variety of experimental conditions, the cAMP secretion rate was directly proportional to intracellular cAMP. This result disagrees with that of Gerisch and Wick (8). They reported a 30-s lag between the changes in intracellular and extracellular cAMP in suspensions of amoebae under conditions in which the level of cAMP was spontaneously oscillating.

The parallel changes in intracellular cAMP levels and the rate of cAMP secretion observed in this study indicate a pseudo-first-order relationship between intracellular cAMP and its transport. This implies that the putative transport mechanism is not saturated at the highest intracellular cAMP levels observed (e.g., during stimulation with 10^{-6} M cAMP). These peak levels are estimated to be 5–10 μM (4, 8). A pseudo-first-order rate constant for the secretion step, K_s, was estimated using the relationship between the measured changes in the intracellular [^{3}H]cAMP/[^{14}C]cAMP ratio and the derivatives of the extracellular [^{3}H]cAMP/[^{14}C]cAMP ratios from the experiments shown in Figs. 2 and 3. (It was assumed that all intracellular cAMP was available for secretion.) The value of K_s was 0.34 min^{-1} and 0.94 min^{-1} for the experiments shown in Figs. 2 and 3, respectively, and did not vary significantly across the time-course of the signaling response. The different rate constants might represent technical or developmental differences (e.g., different levels of transport activity) between experiments carried out on different days rather than a dependence of transport rate on stimulus size.

There was a discrepancy between the between the recovery of intracellular and secreted cAMP. The total [^{3}H]cAMP secreted in response to 10^{-6} M cAMP was equal to ~1.5 × the peak intracellular value. However, when intracellular [^{3}H]-cAMP was at its peak value, the sum of the intracellular and extracellular [^{3}H]cAMP levels (i.e., the total cAMP in the system) was 1.5 × greater than the intracellular cAMP level, equaling the total amount of [^{3}H]cAMP secreted by the end of the response. This simple bookkeeping suggests there is destruction of some [^{3}H]cAMP during the course of the response. The same conclusion was obtained by an analysis of the data in Figs. 3 and 6.

We performed two tests of whether extracellular phosphodiesterases reduced the recovery of secreted [^{3}H]cAMP. In the first, DTT was used to inhibit both secreted and membrane-bound phosphodiesterase activity (2, 9). We compared responses elicited by stimuli of 10^{-6} M cAMP in the presence and absence of 10 mM DTT. Although 10 mM DTT had a slight inhibitory effect on the magnitude of the response, it had little effect on the ratio of intracellular to extracellular cAMP levels. In the second test, we raised the stimulus concentration to 10^{-4} M cAMP to provide greater protection from extracellular phosphodiesterase. However, the relative recovery of intracellular and extracellular cAMP was unaffected by the 100-fold increase in exogenous cAMP. As a further control, [^{14}C]cAMP was routinely added to collection vessels and the [^{3}H]label recovered was normalized for destruction and all other losses after its collection. We conclude that secreted [^{3}H]cAMP was not degraded by extracellular phosphodiesterases nor preferentially lost during subsequent purification in these experiments.

[2] To measure ATP levels after introduction of NaN_3, we used the eight-filter perfusion apparatus. Amoebae that had not been labeled with [^{3}H]adenosine were developed to the early aggregation stage, harvested, gently pipetted onto eight filters (10^6 cells/filter), and synchronized as described in Materials and Methods. Filters were then perfused with NaN_3. Two filters were removed just before the initiation of perfusion with NaN_3, the other six at subsequent time-points. Filters were inverted into 200 μl of 8% formic acid. Later, equal aliquots were applied to cellulose thin-layer plates (Eastman-Kodak, Rochester, N. Y.) and chromatographed (3). The ATP region was scraped and eluted in 1 ml of 100 mM Tris-HCl (pH 7). The ATP content of each sample was determined by the luciferin-luciferase method, employing a scintillation counter to measure light production (13). Values were expressed as a percent of the two control filters removed just before introduction of NaN_3. Within 30 s, ATP levels had fallen to 5%.

Independent of the precise mechanism of cAMP secretion, it was possible to analyze the kinetics of cAMP production from the simultaneous measurements of intracellular and extracellular cAMP levels. We used a simple rate equation:

$$\frac{dI(t)}{dt} = V(t) - \frac{dE(t)}{dt} - K_p I(t) \quad (1)$$

where $I(t)$ = intracellular cAMP level at time t, $V(t)$ = rate of synthesis of cAMP at time t, $E(t)$ = amount of extracellular cAMP by time t, and K_p = first-order rate constant for destruction of intracellular cAMP. It is assumed that phosphodiesterase destruction of intracellular cAMP is first order and all intracellular cAMP is available for secretion and destruction. To estimate K_p, the pseudo-first-order rate constant of phosphodiesterase, we considered the experiment shown in Fig. 6. The decline in the cAMP secretion rate when extracellular cAMP is removed has a time-course identical to that seen when NaN_3 is introduced (see Results). Other data suggest that NaN_3 rapidly blocks the activation of adenylate cyclase (Fig. 7). Therefore, we assumed that at the point of stimulus removal in the experiment shown in Fig. 6, the rate of cAMP production, $V(t)$, dropped abruptly to zero. Eq. 1 can then be solved for K_p in terms of measured quantities.

$$K_p = -\left[\frac{dI(t)}{dt} \Big/ I(t) + \frac{dE(t)}{dt} \Big/ I(t)\right] \quad (2)$$

K_p was calculated according to Eq. 2 employing the data from Fig. 6 for changes in $I(t)$ and $E(t)$ after the stimulus was removed. A plot of $-[dI(t)/dt + dE(t)/dt]$ vs. I(t) was linear; the slope, equal to K_p, was 1.73 min^{-1}. This result supports the premise that phosphodiesterase destruction of cAMP is pseudo-first-order under these experimental conditions.

By assuming that K_p has the same constant value during the response, we calculated $V(t)$, the rate of cAMP synthesis during the course of the signaling response, from Eq. 1. We used the data in Fig. 2 to provide $I(t)$ and $E(t)$ during a 10^{-6} M cAMP stimulus. $dE(t)/dt$, $dI(t)/dt$, and $K_pI(t)$ were calculated from the measured $E(t)$ and $I(t)$ values. $V(t)$, equal to the sum of $dI(t)/dt$, $dE(t)/dt$, and $K_pI(t)$, is illustrated in Fig. 9, along with $K_pI(t)$, $dI(t)/dt$, and $dE(t)/dt$. The calculated rate of cAMP synthesis rose and fell during the continuous 10^{-6} M cAMP stimulus. It is noteworthy that $V(t)$ peaked at ~1–2 min, before the peak in $K_pI(t)$ and $dE(t)/dt$. $V(t)$ then decreased sharply, approaching prestimulus values 3–4 min after the onset of stimulus.

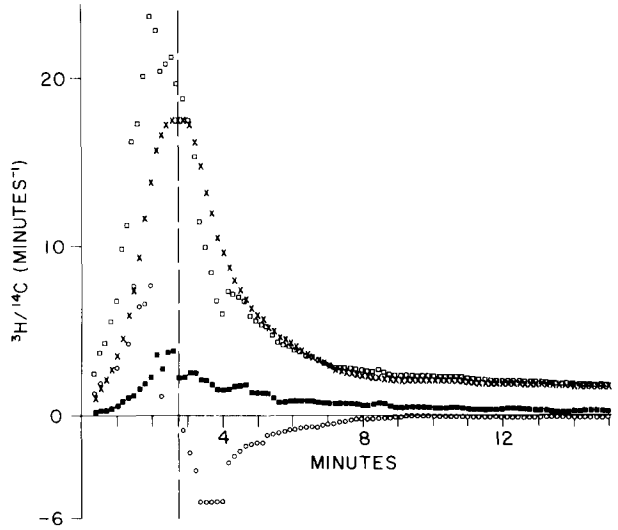

FIGURE 9 Calculated rate of cAMP synthesis during a stimulus of 10^{-6} M cAMP. [^{3}H]cAMP/[^{14}C]cAMP ratios measured for intracellular and secreted cAMP during a 10^{-6} M cAMP stimulus were taken from Fig. 2 and analyzed as described in the text. Eq. 1 was used to calculate the rate of cAMP synthesis, $V(t)$. □, $V(t)$. ×, K_pI (the rate of cAMP degradation). ■, $dE(t)/dt$ (the rate of cAMP secretion). ○, $dI(t)/dt$ (the rate of change in intracellular cAMP). Dashed vertical line passes through the peak of K_pI and $dE(t)/dt$, which occurs 0.5–0.8 min later than the peak in $V(t)$.

To estimate the amount of intracellular phosphodiesterase destruction of cAMP, we computed the areas under the curves for $V(t)$ and $dE(t)/dt$. The value of the former area indicates the total amount of cAMP synthesized, while the value of the latter equals the total amount secreted. The ratio of $[\Sigma E(t)/dt\ \Delta t]/[\Sigma V(t)\ \Delta t]$ equaled ~0.16 by the end of the response to the continuous 10^{-6} M stimulus illustrated in Fig. 2. According to this analysis, 84% of the newly synthesized cAMP was degraded intracellularly and the remaining 16% was secreted. (The fraction of intracellular cAMP secreted can also be calculated by taking the ratio of the first-order rate constant for cAMP secretion to the sum of those for secretion and phosphodiesterase destruction [i.e., $K_s/(K_p + K_s) = 0.34\ min^{-1}/2.07\ min^{-1} = 0.16$].) Analysis of the response to the continuous 10^{-8} M stimulus shown in Fig. 3 led to a similar result. In this case, 53% of the cAMP was degraded intracellularly, whereas 47% was secreted.[3]

The large amount of intracellular cAMP destruction calculated by this approach deserves comment. It is unlikely that newly synthesized [^{3}H]cAMP is destroyed by extracellular phosphodiesterases or preferentially lost during subsequent purification steps, as discussed above. It is possible that the assumption that K_p was constant and independent of cAMP receptor occupancy is not valid. However, the time-course of the rate of cAMP synthesis, $V(t)$ (Fig. 9), calculated using this value for K_p, closely resembles the experimentally determined time-course of adenylate cyclase activation (Fig. 5).

In summary, we have defined the temporal relationship among adenylate cyclase activity, intracellular cAMP levels, and cAMP secretion during cAMP stimulation of *D. discoideum* amoebae. The rate of cAMP secretion is directly proportional to the level of intracellular cAMP. Our data suggest that a significant fraction of newly synthesized cAMP is degraded intracellularly. Nevertheless, the control of the rate of cAMP secretion during a cAMP stimulus appears to be exerted at the level of cAMP synthesis. When a cAMP stimulus was introduced and held constant, adenylate cyclase activity increased for ~1 min, peaked sharply, and then declined to nearly prestimulus levels within 4 min (Fig. 5). These changes slightly preceded the observed changes in intracellular cAMP and closely matched the time-course of $V(t)$ calculated on the basis of the activities of intact cells (Fig. 9). The agreement between these two measures strongly suggests that the activity of adenylate cyclase is the major determinant of cAMP secretion rates in the signaling response.

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[3] The discrepancy between the calculated fractional amount of intracellular cAMP degraded during a 10^{-6} M stimulus and that degraded during a 10^{-8} M cAMP stimulus arises because of the differences in the quantitative relationship between intracellular cAMP and the rate of cAMP secretion (i.e., K_s) in Figs. 2 and 3.

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