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 [3H]cAMP secretion and intracellular [3H]cAMP levels in sensitive D. discoideum amoebae. These data were compared with measurements of the rate of [32p]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 NaN3 was added with the stimulus. During responses elicited by 10^-6 M cAMP, 10^-8 M cAMP, and an increment in cAMP from 10^-6 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 ~10^6 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. 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.

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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.
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. 1 [Difco Laboratories, Detroit, Mich.], 5 g of yeast extract [Difco], 0.962 g of NaH₂PO₄, 7 H₂O, 0.486 g of KH₂PO₄, 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 x 10⁸ cells/ml. Cell density in the stationary phase was 2 x 10⁹ cells/ml. To initiate differentiation by starvation, cells at a density of 2.5 x 10⁶ cells/ml were washed by two centrifugations (600 g for 3 min) at 4°C in M-KK buffer (2.31 g of KH₂PO₄, 1.0 g of K₂HPO₄, 0.5 g of Mg SO₄ per liter, solution pH 6.2), resuspended in M-KK at a density of 2 x 10⁶ 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, and resuspended at a density of 2 x 10⁷ cells/ml. After 20 min at 22°C, the CAMP signaling responses of the cells were synchronized in suspension by adding 5 µl of 10⁻⁴ M CAMP per milliliter of cell suspension every 7 min. The final stimulus was usually 10 µl of 10⁻³ 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, 100 mM EDTA, 0.5 M Tris-HCl (pH 8), and 100 mM DTT and sonicated for 2-5 s (onaBranson Sonifier, model W-140, 50% amplitude, 50 Hz, 20% duty cycle). The sonicate was centrifuged and the supernatant was assayed.

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 [³H]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, for 3-5 min. A stimulus of 10⁻⁴ M CAMP was applied for 2-3 min and the perfusion buffer was then switched back to M-KK, alone. After 8-10 min of recovery, the test stimulus was given, as indicated in Results, and intracellular and extracellular [³H]CAMP were monitored.

PURIFICATION OF CAMP: To quantitatively compare amounts of intracellular and extracellular CAMP, the perfusion technique also rapidly separates secreted CAMP from cells, it permits the simultaneous estimation of intracellular and secreted CAMP.
lular and secreted \[^{3}H\]cAMP, and \[^{14}C\]cAMP (53.1 mCi/mmole, New England Nuclear, Boston, Mass.) was used as an internal standard. Secreted \[^{3}H\]cAMP was collected into 100 \(\mu\)l of a phosphodiesterase stopping solution (8% formic acid, \(10^{-3}\) M \(^{3}H\)cAMP containing 50-80 cpm of \[^{14}C\]cAMP). At each time-point, one filter was removed from the apparatus and inverted into 250 \(\mu\)l of stopping solution containing the same number of counts per minute as the 100 \(\mu\)l 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-\(\mu\)l aliquots of the material extracted from the filters were frozen at \(-20^\circ\)C. After drying under vacuum, the residue was resuspended in 1 ml of M-KK, 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) pre-equilibrated with 50 mM Tris-Cl, 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 \(\mu\)l H.O, 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 reflects 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. 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 \(d/dt(I, \text{extracellular cAMP level}), t, \text{time})\) for purposes of computing adenylate cyclase activity.

**RESULTS**

**Responses Elicited by Continuous Stimuli of High Concentration**

Our initial objective was to relate 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 \((t_{1/2} = 2 \text{ min})\) and slow \((t_{1/2} = 5-6 \text{ min})\) components. Thus, the major portion of the profile had a "sawtooth" appearance 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 \text{ min of stimulation.} It then declined slowly and by 20 \text{ 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 \text{ 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.

**DIAGRAM**

**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 \text{ 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 un-normalized 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.
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 $10^{-8}$ 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 $10^{-8}$ 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.
Inhibition of Adenylate Cyclase Activation by NaN₃

The signaling response is rapidly inhibited by NaN₃ (Fig. 7). When 10⁻⁴ M NaN₃ was added with a 10⁻⁶ M cAMP stimulus, no cAMP secretion was elicited. When 10⁻¹ M NaN₃ 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.

NaN₃ does not antagonize cAMP binding to surface sites, in that the equilibrium binding of 10⁻⁸ M or 5 × 10⁻⁷ M [³H]-cAMP to cells was unaffected by 10⁻⁴ M NaN₃ (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. NaN₃, an inhibitor of oxidative phosphorylation, does cause a rapid reduction in ATP levels in
To measure ATP levels after introduction of NaN₃, we used the eight-542

introduction of NaN₃. Within 30 s, ATP levels had fallen to 5%.

pressed as a percent of the two control filters removed just before

tillation counterto measure light production (l₃). Values were ex-

was determined by the luciferin-luciferase method, employing a scin-

Y.) and chromatographed (3). The ATP region was scraped and eluted

inverted into 200 l of 8% formic acid. Later, equal aliquots were

applied to cellulose thin-layer plates (Eastman-Kodak, Rochester, N.

in these cells (t₁/₂ = 8 s).² However, as illustrated in Fig. 8, NaN₃

had a direct effect on the activation of adenylate cyclase. In this

experiment, 10⁻⁴ M NaN₃ 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. NaN₃ 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 NaN₃.

DISCUSSION

It is evident that the rate of cAMP secretion is closely linked to

the level of intracellular cAMP under a variety of conditions.

² To measure ATP levels after introduction of NaN₃, we used the eight-

filter perfusion apparatus. Amoebae that had not been labeled with

[^3H]adenosine were developed to the early aggregation stage, harvested,
gently pipetted onto eight filters (10⁶ cells/filter), and synchronized as

described in Materials and Methods. Filters were then perfused with

NaN₃. Two filters were removed just before the initiation of perfusion

with NaN₃, the other six at subsequent time-points. Filters were

inverted into 200 l 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 scin-
tillation counter to measure light production (15). Values were ex-

pressed as a percent of the two control filters removed just before

introduction of NaN₃. Within 30 s, ATP levels had fallen to 5%.

The cAMP secretion rate rose and fell in parallel with intracel-

lular cAMP in response to sustained stimuli (Figs. 2-4). Upon

removal of the stimulus, both intracellular cAMP and the cAMP secre-
tion rate declined abruptly (Fig. 6). There is thus no evidence for regula-
tion 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 di-
rectly 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 M (4, 8). A pseudo-

first-order rate constant for the secretion step, Kₚ, was estimated

using the relationship between the measured changes in the

intracellular [[^3H]cAMP]/[^14C]cAMP ratio and the derivatives of

the extracellular [[^3H]cAMP]/[^14C]cAMP ratios from the experi-

ments shown in Figs. 2 and 3. (It was assumed that all

intracellular cAMP was available for secretion.) The value of

Kₚ 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 dif-

ferences (e.g., different levels of transport activity) between

experiments carried out on different days rather than a de-

pendence of transport rate on stimulus size.

There was a discrepancy between the between the recovery

of intracellular and secreted cAMP. The total [[^3H]cAMP se-
creted in response to 10⁻⁶ M cAMP was equal to -1.5 X

the peak intracellular value. However, when intracellular [[^3H]-
cAMP was at its peak value, the sum of the intracellular and

extracellular [[^3H]cAMP levels (i.e., the total cAMP in the

system) was 1.5 X greater than the intracellular cAMP level,
examining the total amount of [[^3H]cAMP secreted by the end of

the response. This simple bookkeeping suggests there is destruc-
tion of some [[^3H]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 phospho-
diestases reduced the recovery of secreted [[^3H]cAMP. In the

first, DTT was used to inhibit both secreted and membrane-

bound phosphodiesterase activity (2, 9). We compared re-
sponses elicited by stimuli of 10⁻⁴ 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 10⁻³ M cAMP to provide greater protection from extracel-
lular phosphodiesterase. However, the relative recovery of

intracellular and extracellular cAMP was unaffected by the

100-fold increase in exogenous cAMP. As a further control,

[^14C]cAMP was routinely added to collection vessels and the

[^3H]label recovered was normalized for destruction and all

other losses after its collection. We conclude that secreted

[^3H]cAMP was not degraded by extracellular phosphodiester-

ases nor preferentially lost during subsequent purification in

these experiments.
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:

$$\frac{dI(t)}{dt} = V(t) - \frac{dE(t)}{dt} - K_p I(t)$$  \hspace{1cm} (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 experiments 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.

$$K_p = - \left[ \frac{dI(t)/dt}{I(t)} + \frac{dE(t)/dt}{I(t)} \right]$$  \hspace{1cm} (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 $-\frac{dI(t)/dt}{I(t)} + \frac{dE(t)/dt}{I(t)}$ 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 $K_p I(t)$ 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 $K_p I(t)$, is illustrated in Fig. 9, along with $K_p I(t)$, $dI(t)/dt$, 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 $\sim$1-2 min before the peak in $K_p I(t)$ and $dE(t)/dt$. $V(t)$ then decreased sharply, approaching prestimulus values 3-4 min after the onset of stimulus.

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 $\Sigma E(t)/dt / \Sigma V(t)/dt$ equaled $\sim$0.16 by the end of the response to the continuous 10$^{-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., $K_s/(K_p + K_s) = 0.34 \times 10^{-6} / 2.07 \times 10^{-6} = 0.16$].) 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 $\sim$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$^{-4}$ 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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REFERENCES

1. Bonner, J. T. 1967. The Cellular Slime Molds. 2nd ed. Princeton University Press. Princeton, New Jersey.
2. Chang, Y. Y. 1968. Cyclic 3',5'-adenosine monophosphate phosphodiesterase produced by the slime mold Dictyostelium discoideum. Science (Wash. D. C.). 160:57-59.
3. Devreotes, P. N., P. L. Derstine, and T. L. Steck. 1979. Cyclic 3',5' AMP relay in Dictyostelium discoideum. I. A technique to monitor responses to controlled stimuli. J. Cell Biol. 80:291-299.
4. Devreotes, P. N., and T. L. Steck. 1979. Cyclic 3',5' AMP relay in Dictyostelium discoideum. II. Requirements for initiation and termination of the response. J. Cell Biol. 80:300-309.
5. Dinauer, M. C., T. L. Steck, and P. N. Devreotes. 1980. Cyclic 3',5' AMP relay in Dictyostelium discoideum. IV. Recovery of the signaling response after cAMP stimulation. J. Cell Biol. 86:545-553.
6. Gerisch, G. 1968. Cell aggregation and differentiation in Dictyostelium. In Current Topics in Developmental Biology. A. Moscona and A. Monroy, editors. Academic Press, Inc., New York. 3:157-197.
7. Gerisch, G., Y. Maeda, D. Malchow, W. Roos, W. Wick, and B. Weser. 1977. Cyclic AMP signals and the control of cell aggregation in Dictyostelium discoideum. In Development and Differentiation in the Cellular Slime Molds. P. Capaccioli and J. M. Ashworth, editors. Elsevier/North-Holland Inc., New York. 105-124.
8. Gerisch, G., and U. Wick. 1975. Intracellular oscillations and release of cyclic AMP from Dictyostelium cells. Biochem. Biophys. Res. Commun. 65:364-370.
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. Hilbrand, F. B. 1956. Introduction to Numerical Analysis. McGraw-Hill, Inc., New York.
11. Julians, M. H., and C. Klein. 1977. Calcium ion effects on cyclic adenosine 3',5'-monophosphate binding to the plasma membrane of Dictyostelium discoideum. Biochim. Biophys. Acta. 497:369-376.
12. Klein, C. 1977. Adenylate cyclase activity in Dictyostelium discoideum amoebae and its changes during differentiation. FEBS (Fed. Eur. Biochem. Soc.) Lett. 88:125-128.
13. LeMasters, J. J., and C. R. Hackenbrock. 1977. Kinetics of product inhibition during firefly luciferase luminescence. Biochemistry. 16:445-447.
14. Loosan, W. 1971. Sensitivity of Dictyostelium discoideum nucleic acid analogues. Exp. Cell Res. 64:484-486.
15. Loosan, W. G. 1975. Dictyostelium discoideum. A Developmental System. Academic Press, Inc., New York.
16. Mullens, I. A., and P. C. Newell. 1978. cAMP binding to cell surface receptors of Dictyostelium. Differentiation. 10:171-176.
17. Pottel, M. J., R. E. Sayre, and S. A. MacKay. 1980. Graphics tools for interactive motion analysis. Computer Graphics and Image Processing. In press.
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., C. Schiermegger, and G. Gerisch. 1977. Adenylate cyclase oscillations as signals for cell aggregation in Dictyostelium discoideum. Nature (Lond.). 266:259-261.
20. Salomon, Y., C. Londos, and M. Rodbell. 1974. A sensitive assay for adenylate cyclase. Anal. Biochem. 58:541-548.
21. Sussman, R., and M. Sussman. 1967. Cultivation of D. discoideum in axenic medium. Biochem. Biophys. Res. Commun. 29:53-55.