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

I. A Technique to Monitor Responses to Controlled Stimuli

PETER N. DEVREOTES, PAMELA L. DERSTINE, and THEODORE L. STECK

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

ABSTRACT

A perfusion technique was developed to deliver [^{14}C]adenosine 3′,5′-cyclic monophosphate (cAMP) stimuli of well-defined magnitude and duration to tritium-labeled *Dictyostelium discoideum* amoebae and simultaneously monitor the elicited secretion of [^{3}H]cAMP (i.e., the relay response). The tritiated compounds secreted in response to [^{14}C]cAMP stimuli were highly enriched in [^{3}H]cAMP and reflected an increase in intracellular cAMP accompanying stimulation rather than the release of a preexisting store or bulk cellular contents. The secretory response (per 10^6 cells) to 2-min stimuli increased during differentiation from about 0.2 pmol at 0.5 h to ~5 pmol of cAMP at 7 h. Without adequate perfusion, amoebae altered the level of cAMP in their environment in two ways: phosphodiesterases destroyed cAMP stimuli under some conditions so as to attenuate the relay response; under other circumstances, secreted cAMP magnified minimal exogenous stimuli into maximal responses. Amoebae, furthermore, would respond to their basal secretion of cAMP autocatalytically if its removal or destruction were interrupted. The perfusion system minimized these cell-induced modifications, allowing control of the level of the stimulus and response in quantitative studies.

KEY WORDS cyclic AMP · *Dictyostelium discoideum* · cellular slime molds · chemotaxis · morphogenesis · secretion

Dictyostelium discoideum grows as free-living soil amoebae which, after a period of differentiation induced by starvation, aggregate to form a succession of multicellular stages (3, 5). Aggregation is mediated by chemotaxis toward the intercellular attractant, cyclic adenosine 3′,5′-monophosphate (cAMP) (1, 11, 13). *In situ* observations suggest that cAMP signals are propagated by relay (2, 17, 18), i.e., cells stimulated by cAMP secrete additional cAMP. It has been demonstrated in vitro that differentiated amoebae secrete cAMP in response to exogenous cAMP stimuli (6, 7, 14, 20). Responding cells are thought to exhibit a temporarily reduced ability to transmit the chemoattractant, based on the observation that signal propagation from aggregation centers is both unidirectional and intermittent (19).

The goal of our studies is the elucidation of the cAMP relay mechanism in *D. discoideum*. In this report, we describe techniques by which stimuli of well-defined magnitude and duration can be delivered to [^{3}H]adenosine-labeled amoebae and the secretion of [^{3}H]cAMP accurately assayed.

A perfusion technique was employed because we recognized that these cells are capable of modifying exogenously applied cAMP stimuli in

two ways. First, an active cell surface phosphodiesterase (PDE) (12) can effectively destroy extracellular cAMP and reduce both the stimulus delivered and the response recorded. Second, by the relay mechanism under study, amoebae can increase extracellular cAMP concentrations. Both of these complications can be minimized by continual, rapid perfusion with defined concentrations of cAMP. In this way, secreted cAMP (and soluble PDE) are removed and the stimulus is constantly renewed. An additional advantage of the perfusion system is that a single preparation can be repeatedly stimulated, washed, and restimulated without mechanical perturbation.

MATERIALS AND METHODS

Media and Buffers

Nutrient-free buffer (NFB), axenic medium (HL-5), bacterial growth medium, nutrient-free agar plates, and nutrient agar plates were prepared as described by Clark et al.[1] TPGA medium is TPG medium (21) with 1.0 g of KH_2PO_4 and 10 g of Casamino Acids per liter. KC medium has been described (21).

Growth and Development

Conditions for the growth of *D. discoideum* strains NC-4 and AX-3 and *D. purpureum* are described elsewhere.[1] Conditions for development on agar substrates or filters followed Clark et al.,[1] except that dimensions were scaled to small numbers of labeled cells. For development on filters, 0.5–1 × 10^6 amoebae in 300 μl of NFB were loaded on 13-mm, 3.0-μm Millipore filters (SSWPO 1300, Millipore Corp., Bedford, Mass.) supported by the base of a Nucleopore "pop-top" filter holder (Nucleopore Corp., Pleasanton, Calif.). After the excess NFB had drained, the filters were placed on 13-mm Millipore adsorbant pads containing 120 μl of NFB. For development on agar, 1–2 × 10^6 amoebae in 20 μl of NFB were deposited on 2 cm^2 2% agar disks which had been dried 30 min at 50°C. The filters and pads or agar disks were then placed in 35-mm plastic Petri dishes (Falcon, Becton, Dickinson & Co., Cockeysville, Md.). For development in suspension, AX-3 cells (10^7 cells/ml in 20–50 ml of NFB) were agitated in a 125-ml Ehrlenmeyer flask at 225 rpm (1 cm radius of gyration). Growth and development occurred at 22°C in a dark incubator.

[^{3}H]Adenosine Labeling

Escherichia coli strain PC0205 (CGSC strain no. 4432), an adenosine auxotroph (*pur A* 45) obtained from Dr. Barbara Bachmann, *E. coli* Genetic Stock Center, New Haven, Conn., was grown to stationary phase in KC broth. The bacteria were diluted 1:50 into TPGA medium containing 10 μg/ml adenosine. After 3 h of incubation at 37°C, 1–5 mCi [^{3}H]adenosine (20–50 Ci/mmol, New England Nuclear, Boston, Mass.) in 200 μl of H_2O was added to 5 ml of bacterial culture. Bacteria were collected after an additional 3 h by centrifugation at 10,000 rpm for 20 min in a Sorvall SS-34 rotor (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.). The bacteria incorporated 30–60% of the [^{3}H]adenosine in the medium. Pellets were resuspended in 1 ml of NFB and stored in 100-μl aliquots in glass culture tubes at −20°C. For labeling, 2–3 × 10^6 amoebae (in <100 μl NFB) were added to 100 μl of the bacterial stock and shaken at 225 rpm at 22°C for 30 min to 3 h. Amoebae were freed of labeled bacteria by three washes with 5 ml of NFB at 4°C. Amoebae took up ~12% of the label per hour.

Perfusion Chamber

Fig. 1 illustrates our perfusion chamber. 10^6 labeled amoebae were placed on the central 1 cm^2 of a 13-mm, 3.0-μm white Millipore filter (SSWPO 1300) resting on a Nucleopore "pop-top" membrane holder. Buffer fell to the filter in 45-μl drops from a height of 0.8 cm and spread to cover the entire surface without overflow. Amoebae were completely submerged for only ~1.5 s as each drop flowed through the filter. The perfusion rate was varied between one drop/2 s and one drop/40 s.

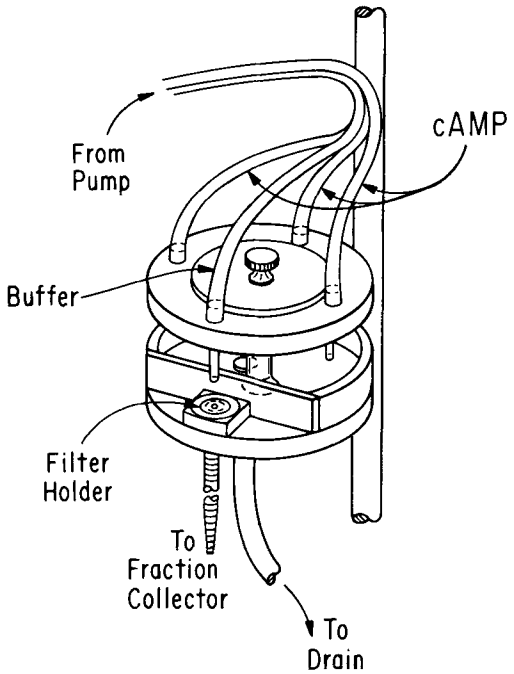

FIGURE 1 Schematic diagram of the perfusion apparatus. The entire apparatus is drawn to the scale of the filter holder which is actually 13 mm in diameter.

[1] R. CLARK, G. RETZINGER, and T. STECK. 1978. Alternative pathways in the morphogenesis of the cellular slime mold, *Dictyostelium discoideum*. Manuscript in preparation.

Solutions were delivered by means of a 4-channel Gilson Minipuls peristaltic pump (Gilson Medical Electronics, Inc., Middleton, Wis.) through "Tygon" tubing (R-3603), the end of which was connected to the tip cut from a 1.0-ml plastic pipette (Falcon). One stream was directed onto the amoebae while three were pumped into a waste receptacle. After passing through the filter, solutions traversed a 3-cm length of polyethylene tubing (Intermedic PE 90, Becton, Dickinson and Co., Clay Adams Div., Parsippany, N. J.). This tubing was connected (through a small piece of Intermedic PE 205) to the tip cut from a 1-ml pipette (Falcon) matched to those above the filter. A drop of buffer emerged from the lower pipette tip as each drop flowed into the filter. Perfusion buffers were changed by manually rotating the disk holding the four pipette tips. The effluent was collected into a M 75 fraction collector (Medical Research Corporation, Boston, Mass.) equipped with a photocell drop counter. The "hold-up" volume of the chamber containing a filter with unlabeled amoebae was measured by switching to a standard solution of [^{3}H]cAMP. After about four drops, the radioactivity in the effluent equaled that of the applied solution. Amoebae likely encountered more abrupt changes in concentration, since most of the mixing volume was located below the filter.

Fractions of 3–12 drops were collected into test tubes containing 20 μl of a stopping solution which was shown to inactivate secreted phosphodiesterase rapidly. This solution contained 0.2 M HCl, 50 mM dithiothreitol (DTT), and 10^{-3} M cAMP. The samples were diluted and neutralized with 1 ml of 5 mM Tris base when purifying cAMP. After an experiment, filters were sometimes removed from the chamber and placed on Millipore pads for development. The number and size of the fruiting bodies, which developed after 24 h on perfused and control filters, were very similar.

Dowex 50W-X4 Chromatography

Dowex 50W-X4 columns (1 ml) were prepared by pouring 2 ml of 50% slurry of Bio-Rad AG 50-X4 (Bio-Rad Laboratories, Richmond, Calif.), preequilibrated in 1 mM Tris-HCl (pH 7.0), into Bio-Rad econocolumns (22). Neutralized samples were decanted onto columns which were then eluted with 1 mM Tris-HCl (pH 7.0). The cAMP peak typically eluted after seven column-volumes. In processing many columns, the first 4 ml of eluate were discarded and the next 5 ml were collected directly into scintillation vials. Samples were evaporated to dryness at 80°C, resuspended in 1 ml of 1% sodium dodecyl sulfate (SDS), and prepared for scintillation counting. The procedure yielded 60–80% recovery of cAMP. Columns were regenerated by addition of 2 ml of 1 N HCl followed by 20 ml of 1 mM Tris-HCl (pH 7.0). Storage in a closed box at 4°C prevented the columns from drying.

Cellulose Thin-Layer Chromatography

1- to 50-μl samples (nine per plate) were applied, with the aid of a drawn-out Pasteur pipette, as small spots on 20 cm × 20 cm cellulose thin-layer plates (Eastman Kodak Co., Rochester, N. Y.). Plates were developed for 8 h by the ascending solvent, acetone: acetic acid:butanol:NH_4OH:H_2O (10:6:14:1:8) (16). 2-cm wide strips extending from each origin were cut out and sectioned into 1.5-cm fractions; these were placed in scintillation vials and eluted with 1 ml of 2% SDS.

Erythrocyte Ghost Binding Assays

Human erythrocyte ghosts, which possess high-affinity cAMP-binding sites (15), were used to isolate [^{3}H]cAMP or to quantitate unlabeled cAMP by isotope dilution.

[^{3}H]cAMP ISOLATION: Samples containing [^{3}H]cAMP in 200 μl of NFB plus 20 μl of 2 M Tris-HCl (pH 7.8) were mixed with 50 μl of packed, freeze-thawed erythrocyte ghosts in 50 μl of 5 mM sodium phosphate (pH 8.0). After incubation for 3–24 h at 4°C in 75 × 12 mm plastic tubes, samples were diluted to 5 ml with NFB at 4°C. Tubes were centrifuged at 3,200 rpm for 20 min and the supernatant fluid was discarded. We have determined the half-time for dissociation of the [^{3}H]cAMP-ghost complex to be 14 h, so that negligible dissociation occurred during the washing step. Pellets were dissolved in 2% SDS, 10 mM Tris-HCl (pH 7.8), and prepared for scintillation counting. Under these conditions, 60% of cAMP was recovered at all cAMP concentrations up to ~10 pmol per assay.

ISOTOPE DILUTION ASSAY: The isotope dilution assay was carried out essentially as described by Hesse et al. (9), except that samples were collected by centrifugation and prepared for scintillation counting as described above.

Scintillation Counting

Liquid scintillation spectroscopy of ^{3}H and ^{14}C was carried out in a Searle Mark II scintillation counter (Searle Diagnostics, G. D. Searle & Co., Des Plaines, Ill.). Triton-toluene fluor was made by mixing 2 liters of toluene fluor (16 g of PPO plus 0.4 g of POPOP per gallon of toluene) with 1 liter of Triton X-100. This mixture was miscible with aqueous samples at a ratio of 7:1. The efficiency for ^{3}H was 60% and for ^{14}C was 50%. During simultaneous counting of both isotopes, spillover of ^{14}C into the [^{3}H]channel was 40% and of ^{3}H into the ^{14}C channel was 0.01%.

RESULTS AND DISCUSSION

Demonstration of the cAMP Relay Response and Analysis of Released Radioactive Material

Typical relay responses are shown in Fig. 2. Amoebae were loaded in the perfusion apparatus and washed dropwise with a stream of buffer at room temperature (22°–24°C). The spontaneous

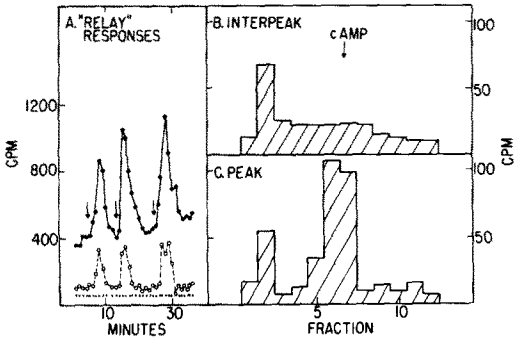

FIGURE 2 Characterization of the secretory response of labeled amoebae. Panel *A*. NC-4 amoebae were labeled for 3 h with [^{3}H]adenosine bacteria and then deposited on agar at 5×10^5 cells/cm^2. After 8 h, 10^6 amoebae were loaded on a Millipore filter in the perfusion chamber and perfused with NFB at 1 drop/30 s for 30 min before the experiment shown. 1-min stimuli of 10^{-7} M cAMP were then introduced (arrows). Aliquots of 20 μl were counted directly (—●—); 40-μl aliquots were analyzed for [^{3}H]cAMP after binding to ghosts (- -○- -) or cAMP-treated ghosts (· · · · ·). Values have been corrected for volume differences but not for cAMP recovery. In an experiment similar to that shown in panel *A*, 50-μl aliquots were taken from fractions collected just before ("interpeak," panel *B*) and just after ("peak," panel *C*) cAMP application and analyzed by thin-layer chromatography.

release of radioactive material was initially high, but subsided during the first 30 min of perfusion. The baseline release rate stabilized at ~0.1% of cellular radioactive material per minute (Fig. 2*A*). Introduction of 10^{-7} M cAMP into the perfusion fluid elicited a rapid and transient two to threefold increase in the rate of tritium release. In other experiments, the increment in cAMP-stimulated tritium release was as low as 20–50% of the background rate.

The following analyses established that the background of unelicited radioactive material consisted of compounds other than cAMP, while the evoked response was attributable primarily to [^{3}H]cAMP secretion.

The released radioactive material was not sedimentable at 10,000 rpm for 30 min or precipitable with trichloroacetic acid in the presence of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), suggesting that the tritium was not carried by cells, organelles, or macromolecules. Furthermore, the number of viable amoebae which escaped through the filter into the filtrate was shown by plaque assay on a bacterial lawn to be <1/min (i.e., $<10^{-6}$ min^{-1}). The release of cells thus accounts for <0.1% of the released tritiated compounds. Background tritium release was fivefold lower at 4°C, consistent with the notion that it reflected a metabolic process.

The selective binding of cAMP to human erythrocyte ghosts provided a convenient way to separate secreted [^{3}H]cAMP from other compounds. Fig. 2*A* illustrates that, after the ghost binding step, the cAMP-stimulated tritium release rate typically increased to a value 3–8 times greater than the background rate. [^{3}H]cAMP binding to ghosts was specific; it could be abolished by preincubating ghosts with 10^{-5} M unlabeled cAMP.

The radioactive compounds contained in peak (Fig. 2*C*) and interpeak (Fig. 2*B*) fractions were analyzed by thin-layer chromatography. Whereas the background (i.e., interpeak) radioactive material was distributed diffusely throughout the chromatogram, the additional radioactive material released during cAMP stimulation (i.e., the peak) was primarily confined to a major spot which cochromatographed with a cAMP standard and a minor spot of slower mobility, corresponding to 5′-AMP (the anticipated breakdown product of cAMP).

In a series of experiments in which amoebae were stimulated with cAMP in the absence of the PDE inhibitor, DTT, the recovery of [^{3}H]cAMP ranged from 40 to 100% of [^{3}H]cAMP plus [^{3}H]5′-AMP. The relative amount of 5′-AMP (presumably reflecting cAMP hydrolysis) could be reduced by adding DTT, increasing the flow rate during perfusion, and reducing the delay between sample collection and chromatography.

The radioactive material collected during the period of elevated tritium release was also examined by Dowex 50 chromatography. A large fraction of this material co-eluted at 7 column-volumes with an authentic cAMP standard; a very small fraction co-eluted with 5′-AMP at 25 column-volumes. The remainder eluted as a peak at 1–2 column-volumes. Most of the unelicited radioactive material also chromatographed in this early peak; thus, there is little spontaneous release of cAMP and 5′-AMP. While the early peak had the chromatographic behavior of ATP, most of it was not altered by digestion with potato apyrase (Sigma) and 5′-nucleotidase (Sigma) under conditions which totally converted an ATP standard to adenosine (which is retained by the column). In other experiments, digestion with a mixture of beef heart PDE (Sigma), apyrase, and 5′-nucleo-

tidase destroyed the late-eluting peaks but had only a slight effect on the early peak.

Cyclic AMP stimulated the appearance of small amounts of labeled compound(s) other than 5'-AMP and cAMP. This compound(s) also cochromatographed with [^{3}H]ATP. Its time course of release differed from that of [^{3}H]cAMP. A careful analysis of the time course of the total tritium vs. [^{3}H]cAMP released in response to cAMP stimuli (see Fig. 3, below) revealed that the fraction of non-cAMP radioactive compound(s) rose at the end of the response. Frequently, this compound(s) initially represented ~10–30% of the total; however, it became a progressively larger fraction as its release persisted beyond that of [^{3}H]cAMP. This material was not 5'-AMP, and its identity is presently under investigation.

A Specific Assay for cAMP Relay

Dowex 50 column chromatography provided a convenient and effective means of purifying [^{3}H]cAMP from sample fractions. As shown in Fig. 3, this step revealed that the cAMP-stimulated release of [^{3}H]cAMP was ~100 times the unstimulated background. A quantitative comparison of the two panels in Fig. 3 indicates that a large fraction of the additional radioactive material elicited by application of exogenous cAMP was [^{3}H]cAMP. After correcting for cAMP losses, 102% in the 12.5-minute peak and 88% in the 19.5-minute peak were found to be [^{3}H]cAMP.

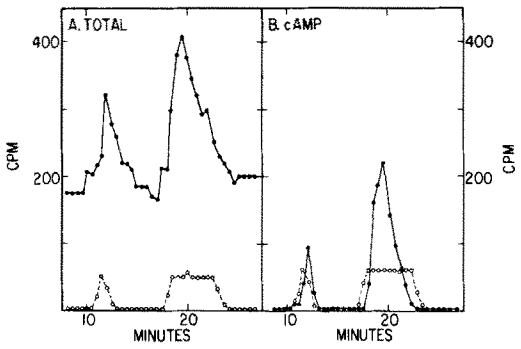

FIGURE 3 Dowex 50W-X4 purification of secreted [^{3}H]cAMP. Panel *A*. NC-4 amoebae were labeled for 60 min and allowed to develop on a Millipore filter for 9.5 h. The filter was placed in the perfusion chamber and washed with buffer at a flow rate of 1 drop/16 s for 25 min before the experiment shown. Initially, the amoebae were stimulated at intervals with 10^{-6} M ^{14}C-cAMP for 2 min. The flow rate was then increased to 1 drop/6 s and amoebae were stimulated as shown. 50 μl of each fraction (~200 μl) were analyzed for total radioactivity. Panel *B*. 100 μl of each fraction collected (panel *A*) were analyzed for [^{3}H]cAMP by Dowex 50W X-4 chromatography. No corrections were made for volume differences or recovery of cAMP. (- -○- -) [^{14}C]cAMP; (—●—) ^{3}H.

Specificity in the cAMP-elicited response was further established by the following observations. No responses to 5'-AMP stimuli were observed in the 10^{-10} to 10^{-4} M range. [^{3}H]cAMP secretion was not elicited by adding 50 mM KCl to the perfusion buffer or by sudden drops in the pH of the perfusion buffer (from pH 6.5 to pH 5.5). Labeled, vegetative amoebae failed to respond. Amoebae, heat-inactivated (80°C, 2 min) before labeling, did not respond to cAMP, nor did [^{3}H]adenosine-labeled *E. coli* alone. Thin-layer chromatographs of 5% TCA extracts of [^{3}H]adenosine-labeled amoebae bore no resemblance to chromatographs of radioactive material released during cAMP stimulation, suggesting that the released material is a selective fraction of the acid-soluble cell contents.

[^{3}H]cAMP relay responses were obtained both from amoebae fed [^{3}H]adenosine directly and from amoebae fed [^{3}H]adenosine-labeled bacteria; the latter labeling technique was somewhat more efficient. Similar responses were observed from amoebae induced to differentiate either at an air-water interface on Millipore filters or on agar, submerged on agar substrates or in suspension. Relay responses were routinely elicited from both NC-4 and AX-3 strains of *D. discoideum* and from *D. purpureum*.

Quantitation of the cAMP Relay Response

While the specific activity of the [^{3}H]cAMP released during relay has not been determined, the magnitude of cAMP secretion by unlabeled amoebae was estimated by isotope dilution assay. As will be demonstrated below (Fig. 6), 10^{-9} M cAMP can elicit a nearly maximal relay response when administered in the presence of 2 mM DTT at a slow perfusion rate. This low level stimulus imposes only a small background against which the relay response can be readily quantitated. As shown in Table I, the amount of cAMP elicited by single 2-min stimuli increased during the starvation period, reaching a maximum at ~8 h for strain AX-3 and at 10–12 h for NC-4. The largest responses were roughly 3–5 × 10^6 molecules/cell for AX-3 and 1–2 × 10^6 molecules/cell for NC-4.

We have also determined levels of intracellular cAMP during a relay response. As seen in Fig. 4,

TABLE I

Quantitation of cAMP Secretion during Development

Strain	Hours of starvation*	Secretion‡ (pmol/10^6 cells)
NC-4:		
Exp. 1	10.0	1.25
Exp. 2	5.0	0.87
	7.5	1.78
	10.0	2.87
AX-3:		
Exp. 1	0.5	0.20
	1.5	0.21
	2.5	1.13
	3.8	1.16
	5.0	1.90
	6.1	3.00
	7.1	4.80
	8.5	3.14

Amoebae were shaken with *E. coli* which had not been labeled with [^{3}H]adenosine (see *[^{3}H]Adenosine Labeling* in Materials and Methods). Differentiation was then initiated by starvation, and the amoebae were deposited on a Millipore filter in the perfusion chamber. The filter was perfused with NFB at a flow rate of 1 drop per 30 s. Four-drop fractions were collected into 20 μl of 2 M HCl. Occasionally, 10^{-9} M cAMP plus 5 mM dithiothreitol (DTT) was added to the perfusion fluid for a 2-min interval. 3–8 such stimuli were delivered at 6–12 min intervals.

Fractions were neutralized with 25 μl of 2 M Tris and cAMP was determined by isotope dilution (Materials and Methods). Backgrounds were estimated by parallel perfusion of a filter without amoebae. Under these conditions, the background was ~0.2 pmol/2 min while maximum stimulated release by AX-3 amoebae was ~7 pmol/2 min.

* NC-4 cells were allowed to develop at 10^6 cell/cm^2 on agar; AX-3 cells were allowed to develop at 10^7 cells/ml in suspension (see Materials and Methods).

‡ Values are averages of 4–6 responses. The cAMP recovered in the fraction in which the stimulus was applied and the two following fractions were taken as the response. (Most cAMP was recovered in the fraction immediately after stimulus application.)

the intracellular level of cAMP in amoebae before experimental stimulation was 1–2 pmol/10^6 cells. (This represents an upper limit; lower values were observed in other experiments.) Stimulation of amoebae with 10^{-9} M cAMP plus 2 mM DTT at a slow perfusion rate elicited an increase in intracellular cAMP levels in both NC-4 and AX-3 over a time course reminiscent of the increased secretion of cAMP shown above. Consistent with extracellular cAMP measurements (Table I), intracellular cAMP pools in AX-3 rose to higher levels than those of NC-4.

Unelicited cAMP Secretion

As shown in Fig. 5, suitably differentiated amoebae respond to slow perfusion with 2–10 mM DTT, an effective PDE inhibitor, by emitting [^{3}H]cAMP secretory responses typical of cAMP relay. The effect was not a function of the DTT

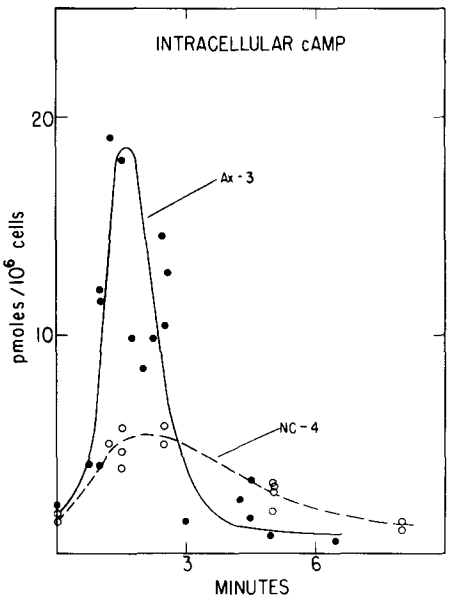

FIGURE 4 Profile of intracellular cAMP concentration during stimulation with 10^{-9} M cAMP plus 5 mM DTT. AX-3 amoebae were allowed to develop for 8 h in suspension at a density of 10^7 cells/ml. NC-4 amoebae were allowed to develop on agar starvation plates for 9 h, washed from the agar with buffer, and adjusted to 10^7 cells/ml. Aliquots containing 10^6 amoebae were deposited on multiple filters resting on Nucleopore "pop-top" membrane holders. Amoebae were washed at room temperature for 5 min at a flow rate of 0.4 ml/min. The perfusion fluid was then replaced by NFB containing 10^{-9} M cAMP plus 5 mM DTT. After various intervals, filters were removed, blotted free of excess liquid, and inverted in 60 μl of 5% TCA in the well of a multiwell culture tray (Falcon). 500 μl of 500 mM potassium phosphate (pH 7.0) was added to each well to neutralize the TCA. Duplicate aliquots of 200 μl were assayed for cAMP by isotope dilution. As controls, extracts were sometimes predigested with 0.01 unit beef heart PDE (Sigma) for 60 min at 37°C. Samples originally containing as much as 7 pmol of cAMP caused negligible inhibition of [^{3}H]cAMP binding in the isotope dilution assay after digestion, indicating little interference by other components in the extracts.

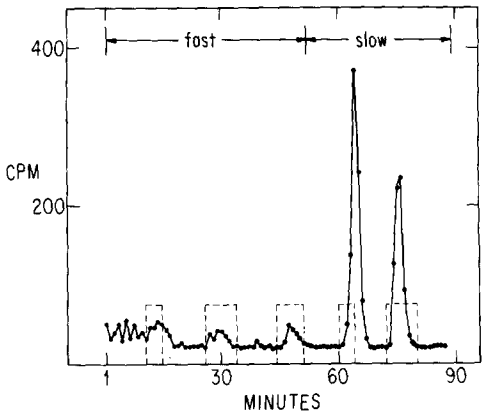

FIGURE 5 Elicitation of cAMP secretion by perfusion with DTT. NC-4 amoebae were labeled for 3 h, allowed to develop on agar for an additional 6 h, placed in the perfusion chamber and washed at a flow rate of 1 drop/5 s for 20 min before the portion of the experiment shown. Amoebae were perfused three times with 2 mM DTT at this rapid flow rate (dashed rectangles). The flow rate was then reduced to 1 drop/30 s, and amoebae were perfused twice with 2 mM DTT (dashed rectangles). During rapid perfusion, 12-drop fractions were collected while 2-drop fractions were collected at the slow flow rate. 10 drops of buffer were added to the latter before cAMP secretion was measured in both sets in parallel.

alone, since rapid perfusion with DTT of the same concentration minimized this response. Addition of 10 mM DTT to AX-3 amoebae in suspension could also elicit immediate increases in total cAMP (M. Dinauer, personal communication).

We interpret this behavior to signify that the amoebae on the filter emit cAMP at low levels in the absence of exogenous stimulation, but that the flowing buffer and cell-bound PDE activity (the soluble PDE being continuously removed by the perfusion stream) prevent responses from building up. Inhibition of PDE allows the spontaneously released cAMP to accumulate and become a stimulus for relay by a highly effective positive feedback loop. The inhibitory effect of rapid perfusion supports this view, since the pool of secreted cAMP at the cell surface would be more rapidly depleted under these conditions, the presence of DTT notwithstanding.

"Clamping" the Extracellular cAMP Concentration

The data presented above imply that a cAMP stimulus not only can be reduced at the cell surface by the action of PDE but also can be magnified by the cAMP secretory response. Before a reliable analysis of the relationship of cAMP dose and response can be established, control of these variables must be secure. The goal, in essence, is to maintain effective cAMP stimulus levels at operator-specified values in the face of complex cellular activity. We call this "clamping," in analogy with the neurophysiological technique (10).

As a means of evaluating the impact of cell-bound phosphodiesterase and the action of the positive feedback loop on the response (and the capability of our perfusion system to minimize these effects), we monitored secretory responses in the presence and absence of DTT, at fast and slow flow rates, and at high and low cAMP concentrations (Fig. 6). Labeled NC-4 amoebae were stimulated with low (10^{-9} M) and high (10^{-6} M) levels of [^{14}C]cAMP in the presence and absence of 2 mM DTT at slow (1 drop/40 s) and rapid (1 drop/5 s) flow rates. Stimulation with 10^{-9} M cAMP elicited a weak response (panel *B*) unless delivered with DTT, which suppressed its destruction (panel *D*), or under rapid perfusion, which constantly renewed the stimulus despite destruction (panel *A*). The relay response to stimuli of 10^{-9} M cAMP was the same in the presence and absence of DTT at rapid flow rates (panels *A* and *C*), confirming that rapid perfusion alone is capable of minimizing PDE destruction of both the input and secreted cAMP.

Stimulation with 10^{-9} M cAMP in the presence of DTT evoked a larger relay response at slow perfusion rates than at rapid perfusion rates (compare panels *C* and *D*). Indeed, the response to slow perfusion with 10^{-9} M cAMP plus DTT approached that elicited by 10^{-6} M cAMP (compare panels *D* and *F*). Control experiments with stimuli of 10^{-6} M cAMP have established that the rate of perfusion does not of itself have an effect on the relay response (compare panels *E* and *F*) and that DTT does not necessarily alter the relay response at this high cAMP concentration (not shown). These results are consistent with the hypothesis stated above. They suggest that, once PDE is inhibited, the cAMP secreted in response to 10^{-9} M cAMP stimuli can act to provoke a maximal response, provided the released cAMP is allowed to act autocatalytically (i.e., at a slow rate of perfusion in the presence of DTT). We conclude that one condition which minimizes modulation of the stimulus size by the cells is a rapid perfusion rate and have adopted 1 drop/4–5 s as a

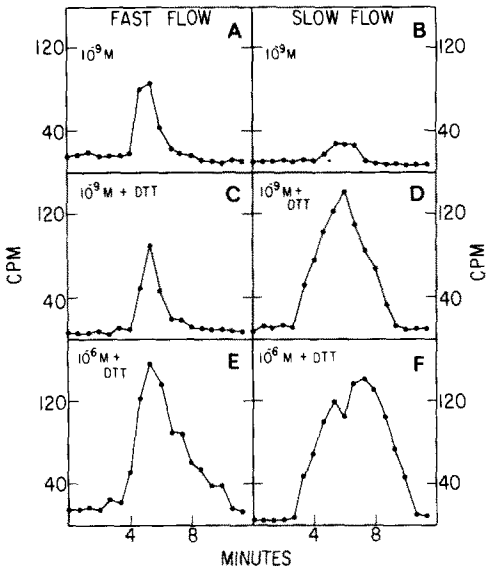

FIGURE 6 The effect of experimental variables on the relay response. Two identical sets of NC-4 amoebae were incubated for 3 h with [^{3}H]adenosine-labeled *E. coli*, except that the labeling of one set was started 1.5 h after the other. [^{3}H]adenosine-labeled amoebae were allowed to develop on agar disks for 6 h after labeling. A 1.5-h experiment was carried out on the first set of amoebae and then an experiment was initiated on the second set. Amoebae were placed in the perfusion chamber and washed with NFB for 15 min before the experiments shown. Amoebae were then stimulated with 10^{-9} M [^{14}C]cAMP, 10^{-9} M [^{14}C]cAMP plus 2 mM DTT or 10^{-6} M [^{14}C]cAMP plus 2 mM DTT as indicated. Not shown are 15 or 20 fractions of low, constant background separating the peaks. The first set of amoebae was perfused at a flow rate of 1 drop/5 s and 8-drop fractions were collected. The second set of amoebae was perfused at a flow rate of 1 drop/40 s and 1-drop fractions were collected. Seven drops of buffer were added to the fractions collected from the second set of amoebae, and both sets of fractions were processed in parallel to isolate [^{3}H]cAMP. At the end of each experiment the filters were counted; the first had 1.4 times the radioactivity of the second. [^{3}H]cAMP counts were normalized accordingly in the figure.

standard flow rate (see accompanying manuscript [4]).

CONCLUSION

The perfusion system (Fig. 1) described in this report offers several advantages in the study of the cAMP relay response in *D. discoideum*. (*a*) Stimuli can be initiated and terminated rapidly and precisely; assay of the [^{14}C]cAMP provides a continuous monitor of the time course of stimulation and the recovery of the cyclic nucleotide. (*b*) Perfusion gives a direct and continuous measure of secretion rate; a background of secreted cAMP never accumulates. (*c*) Soluble phosphodiesterase and other cellular secretions, including cAMP, are not allowed to accumulate and condition the response. (*d*) Amoebae can be washed *in situ* and thus subjected to repeated stimuli without disturbance. (*e*) Most importantly, the ambient cAMP concentration is held fixed by constant renewal, opposing both the tendencies toward cAMP destruction and signal amplification which can otherwise be superimposed by cellular activity. Coupled with the prompt inactivation of PDE in the collected fractions and an efficient cAMP purification step, this system allows a precise delineation of the relay response (e.g., Fig. 3).

The released radioactive material is not a bulk discharge of cytoplasmic contents but a highly specific secretion of cAMP produced after the initiation of the stimulus (Fig. 4). Furthermore, there is no evidence for the accumulation (storage) of cAMP in cells between stimuli. The secretion of cAMP appeared to be a specific physiologic event, in that its magnitude was developmentally regulated (Table I) and differed characteristically for two strains of *D. discoideum*. Furthermore, cAMP secretion could only be elicited by cAMP and not by a number of other types of stimuli.

While cAMP may not be the only molecule secreted by amoebae in response to exogenous stimuli, it is clearly the predominant adenosine-containing secretion (Figs. 2 and 3). The perfusion system employed runs the risk of washing away other, unidentified, secreted molecules which might normally affect the relay response. We found, however, that secretory responses to 10^{-6} M cAMP stimuli were of the same magnitude at high and low perfusion rates (Fig. 6). Furthermore, the kinetics of the response elicited in this system (Table I) resemble those observed previously in a different perfusion system (20) and in suspension (6, 7, 14). Finally, in good quantitative agreement with our data, Gerisch and Wick (8) have reported similar results for strain AX-2.

The relay responses observed were not all the same (i.e., stereotyped) but varied with the magnitude and duration of the stimulus, the perfusion rate, and the presence of the phosphodiesterase inhibitor, DTT (Fig. 6). Two features of the cAMP signal processing apparatus tended to distort responses we sought to characterize by perfusion with defined stimuli. One factor is cell-surface

phosphodiesterase activity. (It is assumed that the soluble PDE is removed by perfusion and that the intracellular PDE does not make contact with extracellular cAMP.) This enzymic activity attenuated responses to low doses of cAMP. Inhibiting the enzyme or replenishing the stimulus through rapid perfusion overcame this effect. Secondly, the cell surface contains the elements needed for a positive feedback loop whereby secreted cAMP compounds itself. When secretions are not rapidly removed by PDE activity or brisk perfusion, responses to subsaturating stimuli can be greatly magnified by this feedback loop. This mechanism can even magnify background cAMP levels into detectable responses (Fig. 5).

The techniques described herein have permitted an accurate and reliable description of the cAMP-stimulated secretion of cAMP in *D. discoideum*. In the following report, the requirements for initiation and termination of this relay response are analyzed in detail.

Addendum

Since the experiments reported here were carried out, several of our methods have been improved by Ms. M. Dinauer. For [^{3}H]adenosine labeling, amoebae are now labeled by adding 3–4 × 10^6 amoebae in 1 ml of NFB to 1–3 aliquots of [^{3}H]adenosine-labeled bacteria. The culture is shaken in a 5-ml beaker at 225 rpm (radius of gyration, 1 cm). For Dowex 50W-X4 chromatography, 6 ml are collected into scintillation vials ([^{3}H]cAMP), and columns are washed twice with 5 ml of HCl between each use.

The authors thank Mr. K. Tomchik for his expert technical 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. Sally Parks for typing. We would also like to thank Mr. A. J. Kittler for constructing the perfusion chamber.

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). P. L. Derstine is a postdoctoral trainee supported by National Institutes of Health Research Service Award GM-7190 from General Medical Science. 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.

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