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Biochemistry

3′:5′-Cyclic AMP-dependent 3′:5′-cyclic GMP accumulation in *Dictyostelium discoideum*

(chemotaxis/desensitization/Ca^{2+}/cellular slime molds/cell aggregation)

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ABSTRACT Suspensions of 3′:5′-cyclic AMP (cAMP)-sensitive cells of *Dictyostelium discoideum* responded to a cAMP pulse with increased 3′:5′-cyclic GMP (cGMP) levels. Under the assay conditions used (2×10^8 cells per ml in 10 mM phosphate buffer, pH 6.0) cAMP (5×10^{-8} M final concentration) increased cGMP levels from 1 pmol per 10^7 cells to 7 pmol per 10^7 cells in 10 sec and basal levels were recovered in 20–25 sec. cGMP accumulation did not occur when cells were in the cAMP-insensitive stage. cAMP-sensitive cells responded with increased cGMP levels when triggered by 5×10^{-8} M 5′-CH_2-cAMP or 10^{-5} M adenosine-5′-methylmonophosphate (5′-AMPMe) but not after addition of 5×10^{-8} M 3′:5′-cyclic IMP (cIMP) or 5×10^{-8} M 5′-AMP. As agonists of cAMP, 5′-CH_2-cAMP and 5′-AMPMe have, respectively, more than 10% and 1% the chemotactic activity of cAMP, while cIMP has 0.01% the activity of cAMP and 5′-AMP is inactive up to a concentration of 10^{-3} M. cAMP-mediated cGMP formation was dependent upon cAMP concentration, with a half-maximal cAMP concentration of about 10^{-8} M. This cAMP concentration agrees closely with that necessary for half-maximal receptor occupation. cAMP-mediated cGMP formation was independent of the presence of extracellular Ca^{2+}; cell aggregation and chemotaxis were also independent of the presence of external Ca^{2+}. Therefore, cAMP action does not depend on stimulation of the Ca^{2+} influx. cAMP was found to mediate desensitization of cAMP-dependent cGMP formation. Additon of 5×10^{-8} M cAMP to sensitive cells induced a desensitization period that lasted 1–5 min. Desensitization was dependent on the cAMP concentration. Finally, we propose that the translation of a chemotactic signal from the cell surface to pseudopod formation in *Dictyostelium* involves changes in the levels of cGMP.

Cell aggregation in the cellular slime molds is mediated by chemotaxis (1). cAMP is the chemotactic molecule (acrasin) in the larger species of *Dictyostelium* (2, 3). In *D. discoideum* the input signal for chemotaxis is a spatial gradient of 3′:5′-cyclic AMP (cAMP) concentration (4) measured by specific cell-surface-bound receptors (5–8). The cAMP–receptor interaction triggers the following two processes necessary for cell aggregation: (*i*) pseudopod formation towards the attractant source (9) and (*ii*) signal amplification or relay (10, 11). Pseudopod formation is achieved within 10 sec after cAMP application (9), while intracellular peaks of cAMP occur at 60 sec and are followed by extracellular peaks 30 sec later (10, 12). The chemotactic response lasts for about 100 sec (13); therefore, the intracellular rise in cAMP does not coincide with the beginning of the chemotactic response but with its end. In an attempt to understand the role of cyclic nucleotides during chemotaxis in *Dictyostelium*, we measured early changes in cyclic nucleotides associated with acrasin addition to pre-aggregative amebae of *D. lacteum*.* In this species cAMP is not used as attractant and there is no evidence for signal relay. Addition of the purified attractant (14) to pre-aggregative amebae of *D. lacteum* induces a 2-fold increase in the intracellular concentration of 3′:5′-cyclic GMP (cGMP) within 10 sec without modifying the cAMP levels.* These results suggest a possible function of cGMP during the chemotactic process. In this study, changes in cGMP associated with cAMP addition have been examined in *D. discoideum*. Our results provide evidence that cGMP is involved in the translation of the chemotactic signal responsible for movement during cell aggregation in cAMP-sensitive and cAMP-insensitive species of *Dictyostelium*.

METHODS AND MATERIALS

Organism. *D. discoideum* NC-4(H) was used for all experiments. Cells were grown on a solid medium and harvested as described (15).

Cell Aggregation. After harvesting, cells were suspended in cold 10 mM phosphate buffer (pH 6.0) in the presence or absence of 1 mM ethylene glycol-bis(β-aminoethyl ether)-N',N'-tetraacetic acid (EGTA) and washed twice in the same buffer. The cell suspension was adjusted to 10^7 cells per ml. Cell aggregation was studied by placing small drops of an amebal suspension on hydrophobic agar (15) buffered with 10 mM phosphate buffer (pH 6.0) to which 1 mM EGTA or 1 mM $CaCl_2$ was added. The amebal drops were incubated at 22° in darkness and the appearance of aggregation centers was monitored at 1-hr intervals.

Assay for Chemotaxis. The chemotactic response of amebae to cAMP was determined as described (16).

cGMP Measurements. Cells were harvested as described above, suspended in phosphate buffer, and starved by shaking (17). After shaking (4 hr if not stated otherwise), cells were centrifuged, washed three times in cold phosphate buffer, and adjusted to 2×10^8 cells per ml. Air was bubbled through for 10 min. Samples of 100 μl were equilibrated for 10 min at 22° under vigorous shaking, after which 20 μl of cAMP (final concentration 5×10^{-8} M if not stated differently) was added. At the time indicated 200 μl of ice-cold ethanol/HCl (60 volumes of ethanol + 1 volume of 11 M HCl) was pipetted into the tubes. After standing 15 min at 0°, the ethanol/HCl extracts (to which 1500 cpm of [8-^{3}H]cGMP had been added) were centrifuged ($8000 \times g$, 2 min), the pellet was washed with 200 μl of ethanol/HCl, and the pooled supernatants were dried at 65°. After drying, samples were dissolved in 500 μl of water and extracted twice with water-saturated ether. The aqueous phase was dried at 65° and then dissolved in 300 μl of 50 mM Tris·HCl buffer (pH 7.5) containing 4 mM EDTA. cGMP recovery was then

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; cIMP, inosine 3′:5′-cyclic monophosphate; 5′-CH_2-cAMP, 5′-deoxy-5′-methylene adenosine 3′:5′-cyclic monophosphate; 5′-AMPMe, adenosine 5′-methylmonophosphate; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N',N'-tetraacetic acid.

* J. M. Mato, F. A. Krens, P. J. M. van Haastert, and T. M. Konijn, unpublished data.

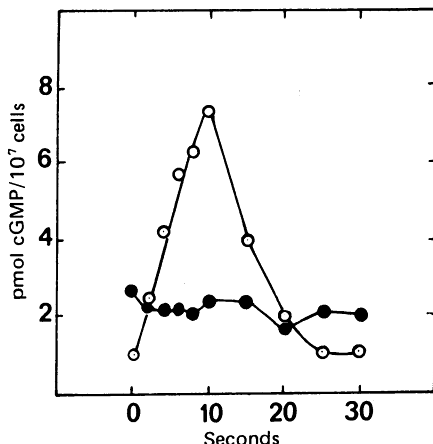

FIG. 1. Time course of cGMP formation after *D. discoideum* cells were triggered with 5×10^{-8} M cAMP. cAMP was added at time zero and the formation of cGMP was followed. (○) Cells starved for 4 hr; (●) cells starved for 0.5 hr.

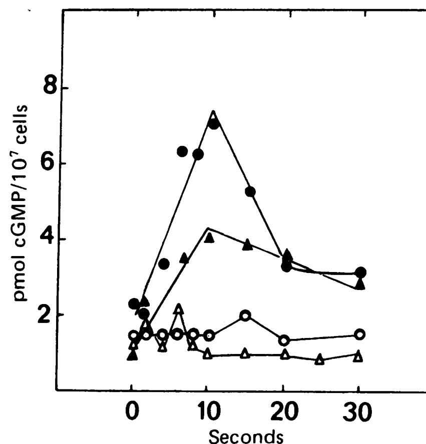

FIG. 2. Effect of different nucleotides as agonists of cAMP-mediated cGMP formation. Cells were starved for 4 hr and stimulated at time zero with: (△) 5×10^{-8} M 5′-AMP; (○) 5×10^{-8} M cIMP; (▲) 5×10^{-8} M 5′-CH_2-cAMP; and (●) 10^{-5} M 5′-AMPMe.

calculated (60–70%) and cGMP levels were measured. cGMP was measured in duplicate by the method of Steiner *et al.* (18) using antibody and [8-^{3}H]cGMP supplied by Amersham (specific activity 20 Ci/mmol). The high specificity of the antibody for cGMP allows cGMP measurements without further sample purification. The amount of cAMP added to the amebae did not show crossreactivity with the antibody. Preincubation of the samples with phosphodiesterase (Boehringer) abolished reactivity with the antibody.

Materials. 5′-CH_2-cAMP was kindly provided by Dr. J. G. Moffatt; adenosine 5′-methylmonophosphate (5′-AMPMe) was a gift of Dr. F. Eckstein; cAMP and 3′:5′-cyclic IMP (cIMP) were purchased from Boehringer; and 5′-AMP and EGTA were from Sigma.

RESULTS AND DISCUSSION

Fig. 1 shows the time course of cGMP formation after addition of cAMP using cells starved for 0.5 hr or 4 hr. In cells starved for 0.5 hr, cAMP addition did not change cGMP levels, while in cells starved for 4 hr cAMP induced, within 2 sec, an increase in the cGMP concentration. cGMP level reached its peak 10 sec after cAMP addition and then fell to the basal level at approximately 20 sec. cGMP levels increased by a factor of 7 when cells were triggered with 5×10^{-8} M cAMP. Basal cGMP levels in the cells starved for 0.5 hr were two to three times higher than in the cells starved for 4 hr. Under our conditions cells reached the aggregation-competence stage after 5–6 hr of starvation. To avoid autonomous cAMP oscillations (12), we used cells starved for 4 hr. At this stage cells respond chemotactically and bind cAMP while cells starved only for 0.5 hr do not bind cAMP and are about 1% as sensitive to cAMP as attractant (5, 6, 19).

To study the specificity of cGMP formation induced by cAMP, we added various nucleotides. In Fig. 2 is shown that while the addition of 5′-AMP or cIMP did not influence cGMP levels, the addition of 5′-CH_2-cAMP or 5′-AMPMe induced an increase in the cGMP levels. 5′-AMP has no chemotactic activity and cIMP has 0.01% the activity of cAMP (3). As a chemotactic attractant, 5′-CH_2-cAMP is slightly less active than cAMP and 5′-AMPMe has about 1% the activity of cAMP (3, 20). The specificity of cAMP-mediated cGMP formation is similar to that of chemotaxis.

Cells washed in phosphate buffer containing 1 mM EGTA and then plated on EGTA/hydrophobic agar started to aggregate within the same time interval (9–10 hr) as they did in the absence of EGTA. The chemotactic sensitivity of cells on EGTA/agar plates was also in the same range (10^{-9}–10^{-8} M) as in control plates. Preincubation of cells with 1 mM EGTA prior to cAMP stimulation does not affect cGMP formation (Fig. 3). These results indicate that extracellular calcium is necessary neither for cell aggregation nor for cAMP-mediated cGMP formation.

In various systems ligand-induced cGMP accumulation depends on stimulation of the Ca^{2+} influx (21). In *D. discoideum* cGMP elevation is independent of external Ca^{2+}, which rules out the above model. Mason *et al.* (22) have reported that cell aggregation in *D. discoideum* depends on extracellular Ca^{2+}; however, we have not been able to confirm their results. The primary action of the cAMP-receptor interaction in *D. discoideum* may be to change the intracellular Ca^{2+} distribution or to increase guanylate cyclase activity without mediation of Ca^{2+}. Other possibilities, such as a direct action on phosphodiesterase, although less likely, cannot be excluded.

As shown in Fig. 4, cGMP formation was dependent on the concentration of cAMP added. Half-maximal cGMP formation occurred at about 10^{-8} M cAMP. This concentration corresponds closely to that which causes half-maximal occupancy of the cAMP receptors and to that present in the background

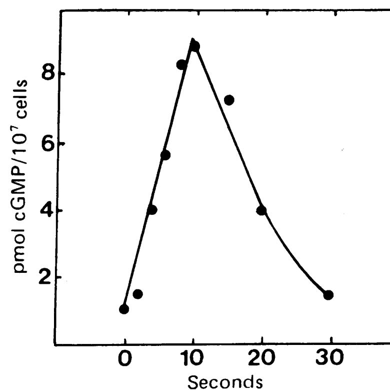

FIG. 3. Time course of cAMP-dependent cGMP formation in the presence of 1 mM EGTA. Cells were starved for 4 hr, washed three times in 1 mM EGTA/phosphate buffer, suspended in the same buffer, and, after 10 min of equilibration, stimulated with 5×10^{-8} M cAMP. Under these conditions the concentration of free Ca^{2+} is below 10^{-7} M (26).

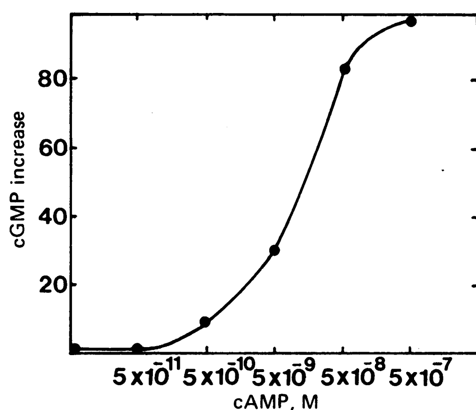

FIG. 4. cAMP concentration dependence of cAMP-mediated cGMP formation. Cells were starved for 4 hr and stimulated with various cAMP concentrations. Samples were taken at 0, 4, 6, 8, 10, 15, 20, and 30 sec after cAMP stimulation. cGMP peaks were obtained at 10 sec. The maximal cGMP increase with respect to basal concentrations was then calculated and plotted, taking the increase obtained with 5×10^{-7} M cAMP as 100.

when cells are triggered by a threshold chemotactic signal (4, 6).

Fig. 5 shows the effect of cAMP when cells are triggered repeatedly. cGMP formation was not induced by a second cAMP pulse (5×10^{-8} M) when given 15, 30, or 60 sec after a first cAMP pulse of the same magnitude. The time necessary for resensitizing the cAMP-mediated cGMP formation was variable, ranging from 1 to 5 min (not shown). When cells were first triggered with a cAMP pulse of 5×10^{-9} M and a second time with a cAMP pulse of $5 \times {}^{-8}$ M, cGMP formation was induced by the second pulse (Fig. 6). Apparently, desensitization of cAMP-mediated cGMP formation is dependent on the cAMP concentration. The existence of a desensitized (refractory) period has been hypothesized to explain unidirectional propagation of the chemotactic signal (23–25). The present results indicate that the period and magnitude of refractoriness depends on the magnitude of the signal. Thus, a given cell may respond within a few seconds to a new signal if the first signal was very weak and, after a stronger signal, may be refractory for as long as 1–5 min.

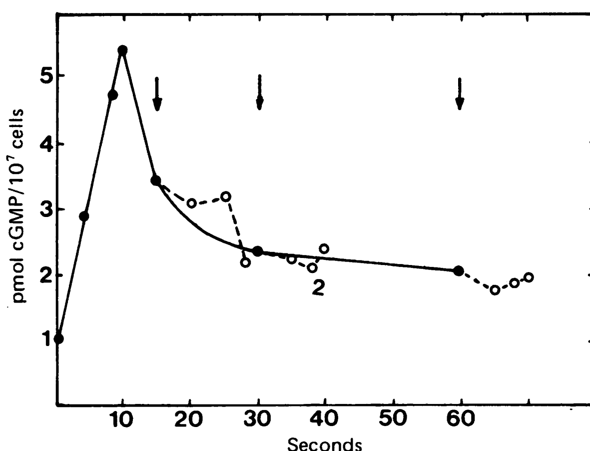

FIG. 5. cAMP-stimulated desensitization of cAMP-dependent cGMP formation. Cells starved for 4 hr were triggered at time zero with 5×10^{-8} M cAMP and the time course of cGMP formation was followed during 60 sec (●). At the time indicated by the arrows (15, 30, and 60 sec) a second cAMP pulse of the same magnitude was given to the cell suspension and the cGMP levels were measured (○). Under these conditions 90% of the added cAMP was hydrolized to 5′-AMP in about 30 sec (data not shown).

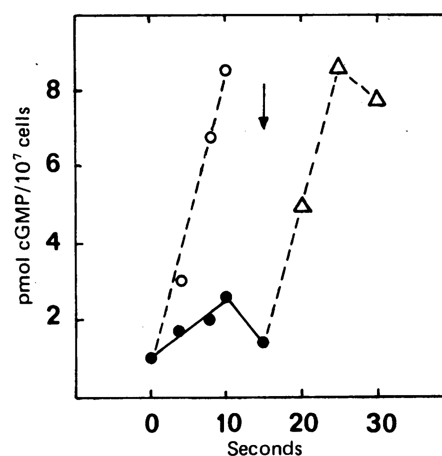

FIG. 6. At time zero cells starved for 4 hr were stimulated with 5×10^{-9} M cAMP (●) or 5×10^{-8} M cAMP (○) and the time course of cGMP formation was followed. At the time indicated by the arrow, cells that had been previously stimulated with a cAMP pulse of magnitude 5×10^{-9} M were triggered again with a cAMP pulse of magnitude 5×10^{-8} M and the time course of cGMP formation was followed (△).

Based on the following observations, we propose that cGMP mediates cAMP-induced chemotactic responses in *Dictyostelium:* (*i*) the increase of the cGMP level precedes pseudopod formation; (*ii*) cGMP levels increased only after the cells were chemotactically sensitive to cAMP; (*iii*) chemotaxis and increase in cGMP show similar nucleotide specificity; (*iv*) a close correlation exists between cAMP receptor occupancy and cGMP increase; and (*v*) the cGMP increase is not limited to cAMP-sensitive* species.

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