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cAMP pulses coordinate morphogenetic movement during fruiting body formation of *Dictyostelium minutum*

(cellular slime molds/multicellular stage/pulsatile signaling/chemotaxis)

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ABSTRACT **Aggregation in the primitive cellular slime mold *Dictyostelium minutum* proceeds by means of chemotaxis toward a continuously secreted folic acid analog [De Wit, R. J. W. & Konijn, T. M. (1983) *Cell Differ.* 12, 205–210]. The onset of culmination is marked by the appearance of concentric waves of cell movement on the aggregate surface. Culmination proceeds by the chemotactic attraction of amoebae to the center of wave propagation, which results in the accumulation of amoebae into a finger-like structure. Evidence is presented that the chemoattractant used during culmination is cAMP, which is secreted in pulses. The cells secrete cAMP themselves; cAMP receptors and phosphodiesterase activity appear on the cell surface just before the onset of culmination. Micromolar concentrations of externally applied cAMP induce disorientation of amoeboid movement at the onset of culmination. These observations are compatible with the hypothesis that the cAMP signaling system organizes multicellular development in both primitive and advanced cellular slime mold species. Advanced species such as *Dictyostelium discoideum* use this signaling system also in an earlier stage of development to organize the process of cell aggregation.**

The cellular slime molds start their life cycle as individual amoebae that feed on bacteria. When bacteria are no longer available, the amoebae aggregate to form a multicellular organism, which ultimately transforms into a fruiting body. Coordinated cell movement is an essential element of both the aggregation process and the process of fruiting body formation. The process of aggregation is well understood, but the process of fruiting body formation is still obscure.

Aggregation takes place by means of chemotaxis. In the simple cellular slime mold species, one cell starts to secrete a chemoattractant continuously, and surrounding cells respond by moving toward the chemoattractant source (1–3). After contact has been made, they start to secrete the chemoattractant themselves. Amoebae of more advanced species such as *Dictyostelium discoideum* secrete their chemoattractant, which has been identified as cAMP, in a pulsatile manner (4–7). Surrounding cells react to this pulse of cAMP by moving chemotactically toward the highest cAMP concentration, and by secreting a pulse of cAMP themselves (relay). These processes result in concentric or spiral waves of inward cell movement, which spread from the aggregation center into the surrounding cells (8). It has been suggested that after aggregation is completed, the morphogenetic movement that ultimately leads to fruiting body formation continues to be mediated by cAMP pulses (9–12). No direct evidence has been presented.

We recently studied the morphogenesis of the simple cellular slime mold *Dictyostelium minutum* (13). This species aggregates by means of continuous secretion of its chemoattractant, which has been identified as a folic acid analog (14).

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Some time after aggregation was completed and shortly before the onset of fruiting body formation, we observed concentric waves of amoeboid movement on the aggregate surface. Cells inside the aggregate were attracted toward the center of wave propagation, which was pushed upward as a consequence. A fruiting body was subsequently formed. These observations suggested that pulsatile chemoattractant secretion might coordinate cell movement at the onset of fruiting body formation. We report an investigation of the characteristics of periodic waves of cell movement in *D. minutum* and present evidence that these waves are the result of chemotaxis toward cAMP, which is secreted in a pulsatile manner.

MATERIALS AND METHODS

Chemicals. cAMP, cGMP, ATP, 5'-AMP, adenosine, folic acid, and snake venom were obtained from Sigma; beef heart phosphodiesterase, from Boehringer Mannheim; [8-^{3}H]cAMP and the cAMP isotope dilution assay, from Radiochemical Centre; DEAE-Sephadex A-25, from Pharmacia; and Instagel, from Packard.

Culture Conditions. *D. minutum* strain 71-2 was supplied by G. Gerisch and cultured in association with *Escherichia coli* 281 on lactose/peptone agar as described (13).

Time-Lapse Cinematography. Time-lapse films of the developing organism were analyzed as previously reported (13). The interval between subsequent frames was 12 s. Magnification on the film was either ×6.3 or ×16.5. The velocity of wave propagation was determined by measuring the time interval needed for a wave to traverse a fixed trajectory on the aggregate surface.

Determination of cAMP Binding Activity and Phosphodiesterase Activity During Development. After being freed from bacteria, 2×10^8 cells were distributed on dishes of 18-cm diameter, which contained 1.5% agar in 10 mM Na/K phosphate buffer (pH 6.5) (P buffer) and incubated at 22°C for 28 hr. Every 4 hr the agar surface of one plate was rubbed gently with a bent glass rod to disperse aggregates or fruiting bodies into small cell clumps. The cells were then harvested and concentrated in P buffer to about 2×10^8 cells per ml for the cAMP binding assay and to 5×10^7 cells per ml for the phosphodiesterase assay. The binding of [^{3}H]cAMP to the cell surface was measured by means of the ammonium sulfate stabilization assay (15). Cells (1.6×10^7) were incubated in 100 μl of P buffer with 10 nM [^{3}H]cAMP (20,000 cpm) and 5 mM dithiothreitol for 1 min at 0°C. Subsequently, 1 ml of saturated ammonium sulfate solution and 50 μl of bovine serum albumin (10 mg/ml) were added. After 5 min at 0°C, the samples were centrifuged for 1 min at $8000 \times g$. The supernatant was removed and the pellet was dissolved in 100 μl of 1 M acetic acid. After addition of 1.5 ml of Instagel scintillation cocktail, the amount of [^{3}H]cAMP was measured by means of an LKB scintillation counter. Assays were performed in triplicate. Nonspecific binding was measured by including 100 μM cAMP in the incubation mixture.

Cell surface-associated cAMP phosphodiesterase activity

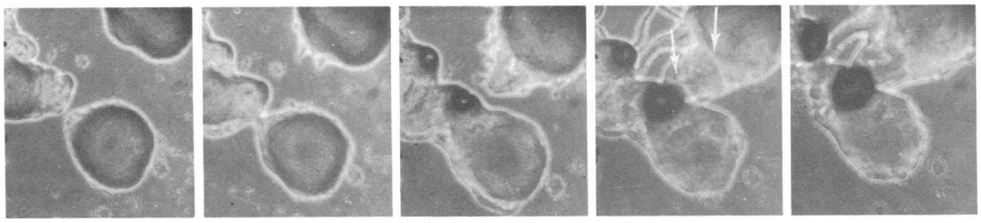

FIG. 1. This series of micrographs copied from a time-lapse film shows at first three aggregates. The aggregate in the upper left corner starts to pulse and attracts streams of amoebae from its two neighbors. The narrow light bands (arrows) are waves that pass over the aggregate surface. (×40.)

was measured by the method of Thompson *et al.* (16), 2×10^6 cells being incubated for 15 min at 22°C in 200 μl of P buffer containing 0.1 μM [^{3}H]cAMP (50,000 cpm). The reaction was terminated by boiling the incubation mixture for 2 min. The boiled samples were incubated for 30 min at 35°C with 50 μg of snake venom (*Ophiophagus hannah*). Nonhydrolyzed cAMP was removed by shaking the incubation mixture for 2 min with 1 ml of Dowex AG 1X2 slurry. After centrifugation, the radioactivity in 0.5 ml of supernatant was determined. Data from the binding assays and the phosphodiesterase assays were standardized to the protein content of the samples, which was measured according to Lowry *et al.* (17).

Measurement of cAMP Secretion During Development. Cells (2×10^8), freed from bacteria, were distributed on a dialysis membrane placed on P buffer agar in Petri dishes of 18-cm diameter. The membrane with the cells was transferred to a dish with DEAE-Sephadex anion-exchange resin in acetate form (pH 6.5) for 4-hr periods: 0–4 hr, 4–8 hr (aggregation), 16–20 hr (tip formation), and 20–24 hr (culmination). Subsequently, the dialysis membrane was removed, and 500 cpm of [^{3}H]cAMP (0.01 pmol) was added to the resin for determination of the cAMP recovery. The resin was transferred to a column, washed with 10 ml of distilled water, and eluted with 0.1 M formic acid. The fractions that coeluted with [^{3}H]cAMP were combined, lyophilized, and dissolved in 100 μl of 50 mM Tris (pH 7.5). The cAMP content was determined as described by Gilman (18) with the standard cAMP assay kit provided by Amersham and was corrected for recovery (which was about 80%).

RESULTS

Characteristics of Pulsatile Signaling in *D. minutum* 71-2. About 4 hr after the completion of aggregation, periodic concentric waves of cell movement were observed to be emitted by a region on the aggregate surface. The resultant movement of amoebae in the aggregate toward this region caused its elevation above the aggregate surface as a tip-shaped structure. This tip continued to emit waves and was lifted higher above the aggregate surface by cells that were attracted by the tip and accumulated underneath. The whole aggregate transformed into a column of cells and finally into a fruiting body (see also ref. 13). An aggregate that started to pulse first may attract streams of amoebae from a neighboring aggregate with which it was not connected before (Fig. 1). This suggests that the emitted signal is a chemoattractant.

At relatively high cell density (about 4×10^5 amoebae per cm^2), it often occurred that, after some aggregates had started to pulse, the majority of the aggregates disintegrated into streams of amoebae. Waves coming from variable directions were observed to pass through these streams. The streams moved at random until the amoebae were exhausted or contracted again into aggregates and formed fruiting bodies. Streams that leave the aggregate are a common characteristic of *D. minutum* (2, 19). The velocity of wave propagation was measured to be between 13 and 23 μm/min. The mean interval between subsequent waves was about 9 min in aggregates and 12 min in streams and did not decrease as signaling continued (Fig. 2) as occurs during *D. discoideum* aggregation.

As putative candidates for the chemoattractant emitted during pulsatile signaling in *D. minutum*, we considered the folic acid analog that mediates aggregation of *D. minutum* (14) and cAMP, the mediator of pulsatile signaling in *D. discoideum*. Because the folic acid analog could not be obtained in sufficiently high quantities, we used folic acid itself, which induced chemotaxis as efficiently as the folic acid analog did in aggregative *D. minutum* amoebae.

As a first step toward identification of the signal, we investigated whether the organization of culmination in *D. minutum* could be overruled by externally applied pulses of folic acid or cAMP. The amoebae were deposited as small populations on a hydrophobic agar surface (20). Droplets of 0.1 μl of 0.1–100 μM folic acid or cAMP were applied 3–6 times with intervals of 10 min, at a distance of *ca.* 100 μm from small populations in the tight aggregate stage. Folic acid had no effect on the process of culmination. After addition of 1–100 μM cAMP, the amoebae streamed out of the aggregate at the moment that tips were formed in control populations, regardless of whether cAMP was applied 1 or 4 hr before tip formation (Fig. 3). The separate streams could form fruiting bodies but only after a considerable delay. cGMP at 1 mM had the same effect as 1–10 μM cAMP; 5′-AMP and ATP applied in concentrations up to 1 mM had no effect.

Amoebae also were allowed to develop on agar that contained cAMP or folic acid (Fig. 4). In the case of folic acid, aggregation was strongly delayed at concentrations above 10 μM. When aggregates were formed, they gave rise to normal fruiting bodies. Aggregation was unaffected with 0.1–1000

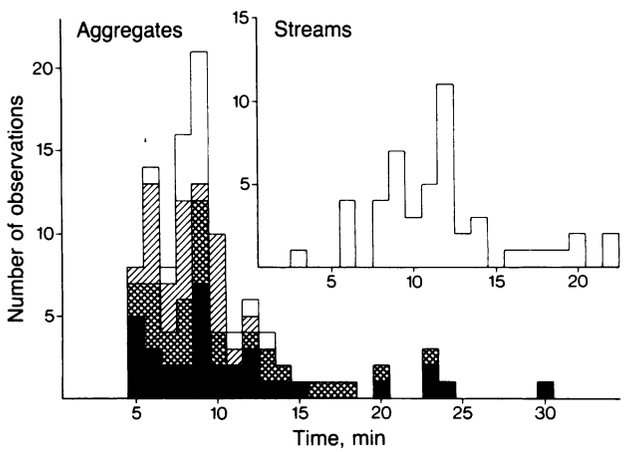

FIG. 2. Frequency distribution of intervals between subsequent waves in aggregates and streams. The darkest shade represents the intervals between the first three waves. Every lighter shade represents intervals between the second three waves, third three waves, etc. The mean interval in streams is somewhat larger than that in aggregates.

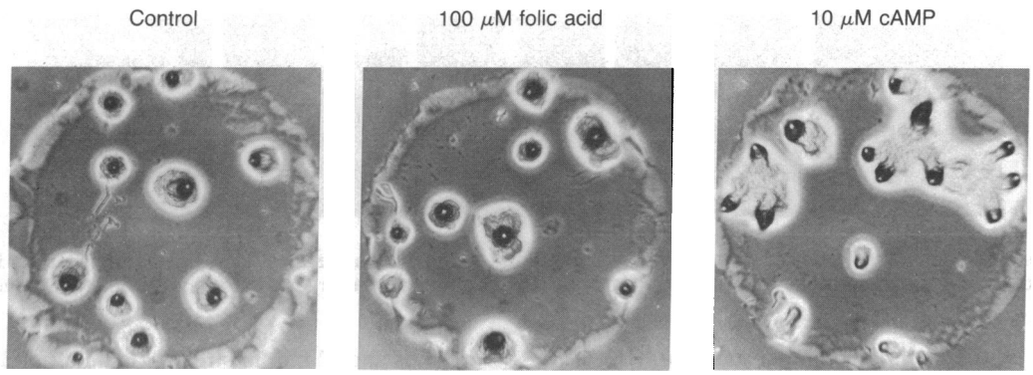

FIG. 3. Pulses of cAMP applied to *D. minutum* aggregates in small populations induce the streaming of amoebae out of the aggregate. Pulses of folic acid have no effect. (×50).

μM cAMP in the agar. However, no tips were formed at 10 μM and higher concentrations of cAMP; the aggregates disintegrated into streams, which moved at random until the amoebae were exhausted, a process that also may occur in crowded populations. The fact that, during *D. minutum* development, cAMP only interferes at the moment that pulsatile signaling would become effective and in a manner that suggests overruling of the signal strongly suggests that the signal itself is cAMP.

cAMP Receptors and Cell Surface-Associated cAMP Phosphodiesterase Activity During Development. In order to obtain more evidence for the involvement of cAMP in pulsatile signaling in *D. minutum*, we determined whether cell surface cAMP receptors and cyclic nucleotide phosphodiesterase, two essential elements of intercellular communication mediated by cAMP (for a review see ref. 21), appear during *D. minutum* development. The results are presented in Fig. 5. The phosphodiesterase activity appeared after the comple-

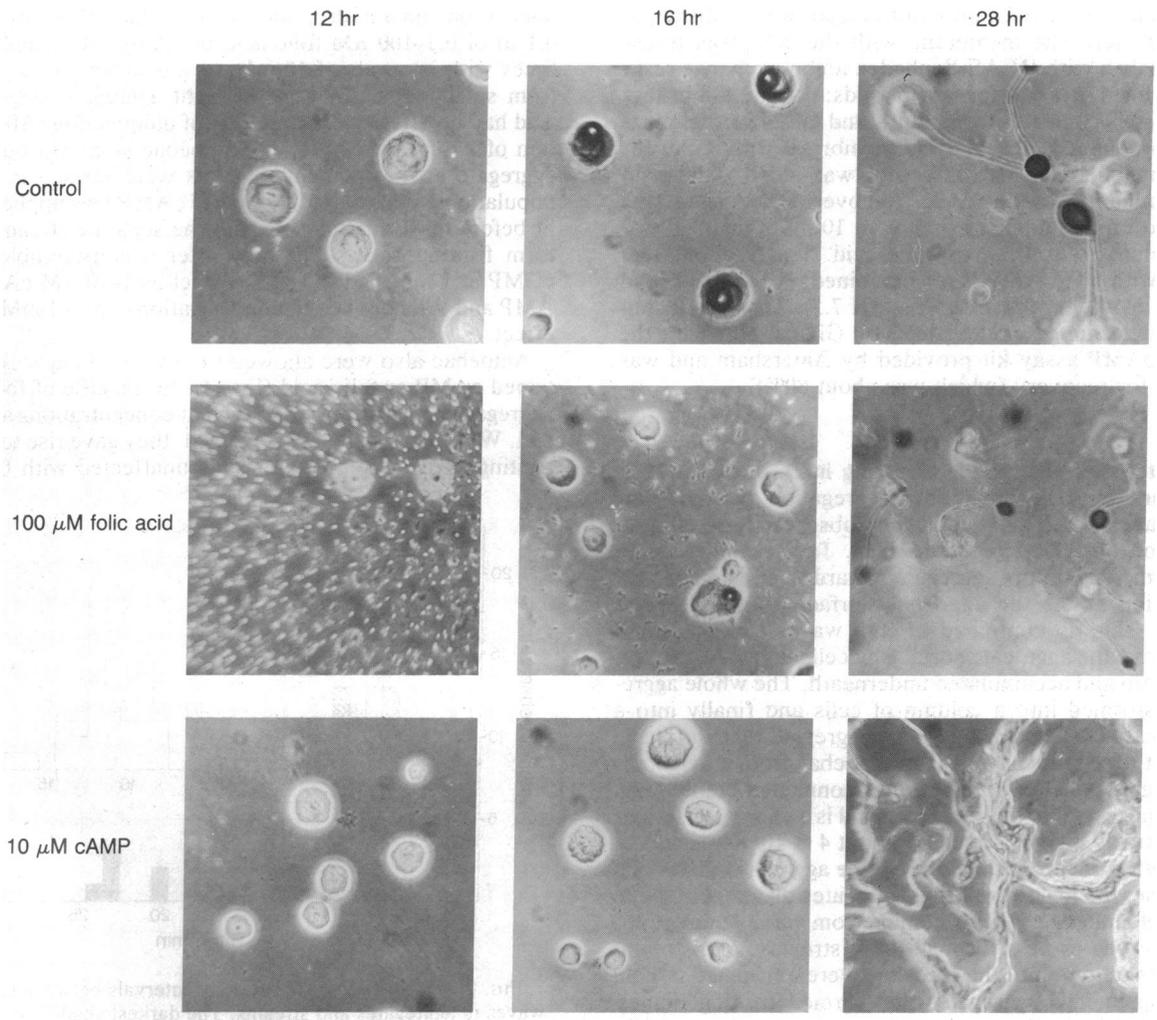

FIG. 4. Development of *D. minutum* on nonnutrient agar that contains cAMP or folic acid. Folic acid induces a delay of aggregation, but culmination proceeds normally. cAMP does not affect aggregation, but tip formation fails to take place; instead, the aggregate disintegrates into streams of amoebae. (×60.)

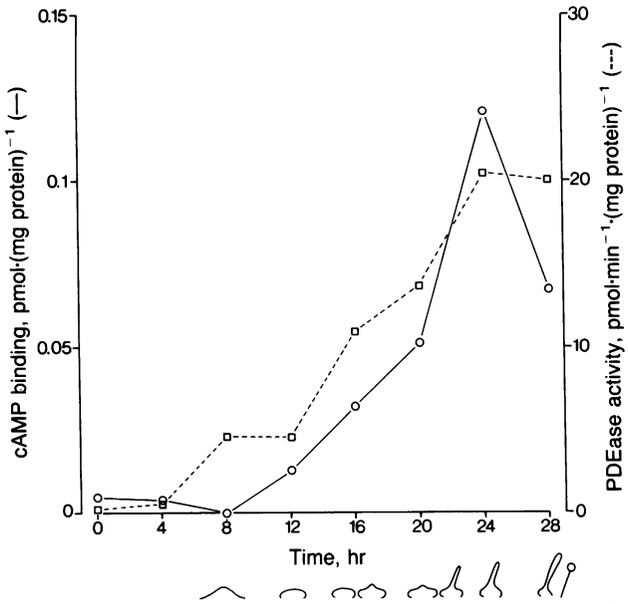

FIG. 5. Cell surface associated-cAMP binding activity and phosphodiesterase (PDEase) activity during development of *D. minutum*. Aggregation is completed at 8 hr, pulsatile signaling starts between 12 and 16 hr, tips are being formed at 16 hr, and, within the following 16 hr, culmination reaches completion. The mean values of three experiments are presented.

tion of aggregation and reached its maximal level during culmination. cAMP binding sites were first evident in the tight aggregate stage (12 hr) and also increased to a maximum during culmination. The analysis of cAMP binding at different cAMP concentrations resulted in a curvilinear Scatchard plot (Fig. 6), which may point to two classes of receptors with different affinities or to negative cooperativity (22, 23). There are about 200 apparent high-affinity sites (α) per cell with a K_d of 14×10^{-9} M and about 8000 apparent low-affinity sites (β) with a K_d of 4×10^{-7} M. The apparent dissociation constants are very similar to those reported for cAMP binding to aggregative *D. discoideum* cells, but the number of binding sites per cell is considerably lower.

In order to exclude the possibility that the observed cAMP binding is due to binding to cell surface phosphodiesterase, we determined the specificity of cAMP binding and of phosphodiesterase. About 1000-fold higher concentrations of cGMP than cAMP were required to obtain equal inhibition of [^{3}H]cAMP binding, while only 2- to 5-fold higher cGMP concentrations were sufficient to inhibit the hydrolysis of [^{3}H]cAMP to the same extent as exhibited by cAMP. Similar results have been obtained in *D. discoideum* (15, 24). AMP, ATP, and adenosine did not inhibit cAMP binding.

Secretion of cAMP During Development. Other evidence for the role of cAMP during multicellular development would be the demonstration of its secretion during the later stages. We realized that the detection of cAMP secretion might be difficult because of the high phosphodiesterase activity that appears after aggregation. Therefore, we tried to withdraw cAMP from phosphodiesterase activity by distributing the cells on dialysis membrane and placing the membrane on an anion-exchange resin that binds cAMP and prevents its further diffusion and hydrolysis.

We found no significant differences between the amount of cAMP secreted before and during aggregation and the amount of cAMP secreted during tip formation and culmination. (In all cases, 2×10^8 cells secreted about 25 pmol of cAMP over 4-hr periods.) This inability to measure an increase in cAMP secretion in the multicellular stage is most likely due to the high phosphodiesterase activity that appears in this stage and to the fact that cAMP secreted by a field of amoebae is in a much better position to diffuse through the membrane than is cAMP secreted in the constricted volume of the aggregate. The experiments demonstrate, however, that *D. minutum* amoebae are capable of synthesis and secretion of cAMP.

DISCUSSION

Movement of amoebae during culmination of *D. minutum* appears to be coordinated by the pulsatile secretion of chemoattractant from a region that later forms the tip of the rising fruiting structure. Externally applied pulses of relatively low concentrations of cAMP cause disorientation of amoeboid movement at the moment that pulsatile signaling would become visible. The effect of cAMP cannot be mimicked by AMP, ATP, or folic acid, an analog of the acrasin of *D. minutum* (14). cGMP can mimic the effect of cAMP at 100- to 1000-fold higher concentrations. The cells secrete cAMP, and at the onset of pulsatile signaling, phosphodiesterase activity and cAMP-specific binding sites appear on the cell surface. The characteristics of cAMP binding activity in *D. min-*

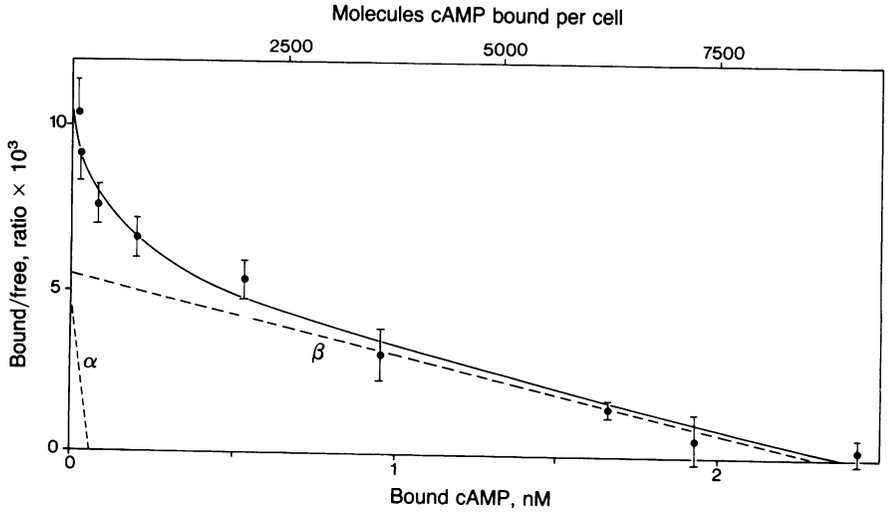

FIG. 6. Scatchard plot of the cAMP binding activity of cells in the early culmination stage. Binding was assayed at 1, 3, 10, 30, 100, 300, 1000, 3000, and 10,000 nM cAMP. The curvilinear shape of the plot points to negative cooperativity or to two receptors with different affinity. —, Least-squares fit of the data to a hyperbolic function; – – –, calculated Scatchard plots for two apparent receptors with different affinity. Data are shown as the mean and SEM of three experiments.

utum are similar to those of the chemotactic cAMP receptor used during aggregation of *D. discoideum*. We conclude from these data that the primitive species *D. minutum* most likely uses the same cAMP signaling system to organize the process of culmination that *D. discoideum* and other advanced species use to organize the process of aggregation.

Evidence that pulsatile signaling also functions during *D. discoideum* culmination comes from Durston *et al.* (10), who reported that the upward movement of the *D. discoideum* culminating structure proceeds with regular intervals of 6 min. They occasionally observed concentric waves of cell movement at the onset of culmination. The early culmination stage of *D. discoideum* is, as in *D. minutum*, very sensitive toward disturbance by externally applied cAMP (25). We recently found that cAMP receptors and cell surface phosphodiesterase show a significant second increase at the onset of culmination in *D. discoideum* (unpublished results); the first increase occurs during aggregation and is followed by a decrease in the slug stage (22). cAMP levels show a similar second increase during culmination (26, 27). This probably means that the cAMP signaling system functions during at least two periods in the development of *D. discoideum*.

D. minutum 71-2 forms more regularly shaped fruiting bodies than do other *D. minutum* strains and exhibits its pulses most clearly. However, the size of the cell mass, which can be organized into a single fruiting body, is very small if compared to *D. discoideum* (28). Aberrations such as streaming (strains 71-2, V3, and 39) and bifurcations of the culminating structure (strains V3 and 39; ref. 13) are probably also a consequence of the relatively low capacity of the cAMP signaling system in *D. minutum* to entrain a group of cells into a single structural unit. The low velocity of wave propagation (1/20th that in *D. discoideum*; ref. 29), and the small number of cAMP receptors also may be related to this low organizing capacity.

D. discoideum acquires full competence for cAMP signaling about 6 hr after the removal of food and then starts to aggregate (19). *D. minutum* starts to aggregate almost immediately after the removal of bacteria and uses an acrasin, whose chemical structure is very close to folic acid (14), the attractant secreted by bacteria. It is likely that *D. minutum* requires no interphase because the same detection system is used for aggregation as for food seeking. In the primitive *D. minutum* species, the cAMP signaling system is developed on behalf of the process of culmination. Characteristic of the more advanced *D. discoideum* is the fact that not only the organizing capacity of cAMP signaling is much larger but also the system is used in an earlier stage of development to regulate the process of aggregation.

Polysphondylium species, which use glorin as chemoattractant during aggregation (30), also seem to shift to cAMP signaling at a later stage in the life cycle (31, 32).

All above mentioned evidence supports the hypothesis that the cAMP pulsatile signaling system is the characteristic organizer of morphogenesis in *Dictyostelium* and *Polysphondylium*.

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