cAMP pulses coordinate morphogenetic movement during fruiting body formation of Dictyostelium minutum
Schaap, Pauline; Konijn, Theo M.; Haastert, Peter J.M. van

Published in:
Proceedings of the National Academy of Sciences

DOI:
10.1073/pnas.81.7.2122

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1984

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
cAMP pulses coordinate morphogenetic movement during fruiting body formation of Dictyostelium minutum

(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. Cullmination 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 chemooattractant 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 chemoattrantant continuously, and surrounding cells respond by moving toward the chemoattrantant source (1–3). After contact has been made, they start to secrete the chemoattrantant themselves. Amoebae of more advanced species such as Dictyostelium discoideum secrete their chemoattrantant, 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 chemoattrantant, which has been identified as a folic acid analog (14). 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 chemoattrantant 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; [8-3H]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 × 10^6 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 × 10^6 cells per ml for the cAMP binding assay and to 5 × 10^7 cells per ml for the phosphodiesterase assay. The binding of [3H]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 [3H]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 × 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 [3H]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...
was measured by the method of Thompson et al. (16), 2 x 10^6 cells being incubated for 15 min at 22°C in 200 μl of P buffer containing 0.1 μM [3H]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 x 10^6), 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 [3H]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 [3H]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 chemotactant.

At relatively high cell density (about 4 x 10^6 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). Dropkets of 0.1 μl of 0–100 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 μM of cAMP.
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.

**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).

**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.)

**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-
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 $14 \times 10^{-9}$ M and about 8000 apparent low-affinity sites (β) with a $K_d$ of $4 \times 10^{-7}$ 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 $[^3H]$cAMP binding, while only 2- to 5-fold higher cGMP concentrations were sufficient to inhibit the hydrolysis of $[^3H]$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, $2 \times 10^8$ 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. minitum amoebae are capable of synthesis and secretion of cAMP.

**DISCUSSION**

Movement of amoebae during culmination of D. minitum appears to be coordinated by the pulsatile secretion of chemotacticant 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. minitum (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. minitum...
utum are similar to those of the chemotactic cAMP receptorused during aggregation of D. discoideum. We concludefrom these data that the primitive species D. minutum mostlikely uses the same cAMP signaling system to organize theprocess of culmination that D. discoideum and otheradvanced species use to organize the process of aggregation.

Evidence that pulsatile signaling also functions during D.discoideum culmination comes from Durston et al. (10), whoreported that the upward movement of the D. discoideumculminating structure proceeds with regular intervals of 6min. They occasionally observed concentric waves of cellmovement at the onset of culmination. The early culminationstage of D. discoideum is, as in D. minutum, very sensitive
toward disturbance by externally applied cAMP (25). We re-cently found that cAMP receptors and cell surface phospho-diesterase show a significant second increase at the onsetof culmination in D. discoideum (unpublished results); thefirst increase occurs during aggregation and is followed by a
decrease in the slug stage (22). cAMP levels show a similarsecond increase during culmination (26, 27). This probablymeans that the cAMP signaling system functions during atleast two periods in the development of D. discoideum.

D. minutum 71-2 forms more regularly shaped fruitingbodies than do other D. minutum strains and exhibits itspulses most clearly. However, the size of the cell mass, whichcan be organized into a single fruiting body, is verysmall if compared to D. discoideum (28). Aberrations such asstreaming (strains 71-2, V3, and 39) and bifurcations of theculminating structure (strains V3 and 39; ref. 13) are prob-ably also a consequence of the relatively low capacity of thecAMP signaling system in D. minutum to entrain a group ofcells into a single structural unit. The low velocity of wavepropagation (1/20th that in D. discoideum; ref. 29), and the smallnumber of cAMP receptors also may be related to this loworganizing capacity.

D. discoideum acquires full competence for cAMP signal-ing about 6 hr after the removal of food and then starts toaggregate (19). D. minutum starts to aggregate almost imme-diately after the removal of bacteria and uses an acrasin,whose chemical structure is very close to folic acid (14), theattractant secreted by bacteria. It is likely that D. minutumrequires no interphase because the same detection system isused for aggregation as for food seeking. In the primitive D.minutum species, the cAMP signaling system is developedon behalf of the process of culmination. Characteristic of themore advanced D. discoideum is the fact that not only theorganizing capacity of cAMP signaling is much larger butalsoso the system is used in an earlier stage of development toregulate the process of aggregation.

Polysphondylium species, which use glorin as chemoo-attractant during aggregation (30), also seem to shift to cAMPsignaling at a later stage in the life cycle (31, 32).

All above mentioned evidence supports the hypothesis thatthe cAMP pulsatile signaling system is the characteristicorganizer of morphogenesis in Dictyostelium and Polysphondy-lyum.

P.V.H. was a recipient of a fellowship from the Foundationfor Fundamental Biological Research (BION) which is subsidizedby the Netherlands Organization for the Advancement of BasicResearch.