Intracellular oscillations and release of cyclic AMP from Dictyostelium cells

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SUMMARY: Cyclic AMP is periodically released as an intercellular signal from cells of Dictyostelium discoideum in the course of their differentiation from the growth phase to the aggregation-competent stage. The pulsatile release is based on the sharp increase of intracellular cyclic AMP by a factor of about 10. Both the temporal relation of intracellular and external cyclic-AMP spikes and the quantitative aspects indicate that oscillatory activation of adenylate cyclase is an important feature of the signal generating system that controls development of D.discoideum.

Cells of the slime mold, Dictyostelium discoideum, aggregate in response to chemotactic signals. In cell layers the attractant is often released from aggregation centers in periodic pulses which are then relayed in from of waves over a territory covered with cells. Cyclic AMP acts as a chemotactic factor as well as a trigger for wave propagation (1, 2, 3). Periodic activities reminiscent of signal generation in cell layers can be observed in cell suspensions (4). These activities are correlated with oscillations of extracellular cAMP concentrations (5). They are amenable to biochemical analysis because of the high degree of synchroneity obtained in stirred suspensions. The results presented in this paper indicate that a primary event during a signalling phase is an increase of the intracellular cAMP level which is followed by the rise of extracellular cAMP.

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Methods

Cells of the Ax-2 strain, clone 110, were cultivated in growth medium supplemented with 18 g maltose per liter (8). Clone 110 is a fast differentiating strain that in suspension starts spontaneous oscillation already 4 to 5 hours after the end of growth. The cells were harvested at a density of 4×10^6 per ml, washed with 17 mM phosphate buffer p_H 6.0, and shaken in a suspension containing 1×10^7 cells/ml. 5.5 hours later the cells were washed once with buffer, suspended at a density of 2×10^8 per ml and bubbled with oxygen for the recording of periodic light scattering changes (4). Simultaneously, samples were taken for the determination of intra- and extracellular cAMP.

The high activity of cell-surface phosphodiesterase (7) demanded an improved technique for instantaneous separation of cells and supernatant, combined with rapid enzyme inactivation. The technique used is shown in fig. 1. Intracellular cAMP concentrations are expressed in µmoles per liter of densely packed cell sediment, obtained by centrifugation of the cells for 5 minutes at 1000 x g into calibrated capillaries. 1 µl sediment corresponded to 1.3×10^6 cells and to 88 µg protein as determined according to Lowry. The sediment volume of the cell suspension used for the experiment shown in fig. 2 was 17 per cent v/v.

To 50 µl samples both of cell extract and supernatant 0.1 M HCl was added and TCA was extracted 5 times using 150 µl ether. Then veronal-acetate solution was added so that the mixture finally contained Michaelis buffer p_H 4.0. cAMP was determined using the Gilman binding assay (9). Controls were taken for recovery of cAMP and for inhibition of binding. Under the experimental conditions, and for the volumes of supernatant used (20 µl per 200 µl), the apparent cAMP-values were somewhat higher than

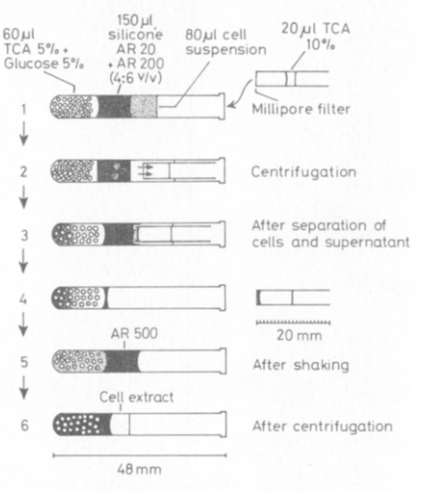

Fig. 1. Separation of cells from supernatant and inactivation of cAMP-degrading enzymes.
(1) A mixture of silicone AR 20 and 200 (Wacker Chemie, München), is layered in microcentrifuge tubes over glucose-loaded TCA which is filled up by 0.5 mm glass beads. Polyethylene tubes closed at one end by Polyvic Millipore filters, 0.6 µm pore size, are prefilled with TCA under avoidance of contact between TCA and the filter disc. (2) After layering of cell suspension on the silicone, the filter tube is rapidly fitted in and separation accomplished by centrifugation in a Beckman 152 microcentrifuge. Separation occurs within less than 2 seconds of centrifugation. (3) After 7 seconds of centrifugation the balls of densely packed cells that have passed the silicone are dispersed between the glass beads, and a small layer of silicone has closed the filter tube. (4) The latter is pulled out and the volume of its contents is measured. In the outer tube the rest of the supernatant and most of the silicone is sucked out and (5) replaced by AR 500 which has a higher density. Using a whirlmix both the water and the silicone phases are shaken but not intermixed. (6) After centrifugation the clear cell extract lies on top of the silicone layer which contains both the glass beads and cell debris.

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under standard conditions, and the values were corrected accordingly. The difference of the peak values compared to the uncorrected ones was less than 20 per cent. From cell extracts, smaller sample volumes were used so that correction was unnecessary. Degradation of the material by phosphodiesterase from beef heart (Boehringer, Mannheim, 0.15 units per ml, 30 minutes at 36^{o}C) was checked.

Results and Discussion

Light scattering measurements in cell suspensions indicate that during the interphase between growth and full aggregation-competence D.discoideum cells develop the ability to undergo synchronous periodic activities (4). Fig. 2 shows that the light scattering responses are associated with sharp rises of both the intracellular and the external cAMP concentrations. The temporal pattern of the oscillations has the following characteristics:

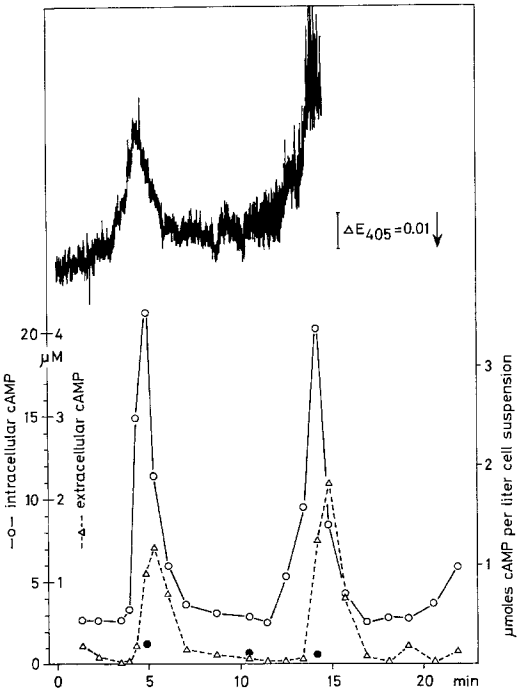

Fig. 2. Periodic light scattering changes (top) and cAMP oscillations (bottom) in a cell suspension.
Left ordinate: intra- and extracellular concentrations of cAMP. Right ordinate: quantities of cAMP in either the intracellular or extracellular compartment. The difference between the right ordinate and the left ones is due to the different volumes of cell mass and extracellular space (see Methods section). Filled circles: Intracellular cAMP after incubation of the extracts with phosphodiesterase. The removal of samples reduced the volume of the cell suspension in the optical cuvette so that light scattering became noisy at the end of the experiment.

(1) The intracellular cAMP concentration rises sharply within 1 to 2.5 minutes. In the present experiment the increase was 8 fold, and in another experiment up to 11 fold. Intracellular cAMP-oscillations of similar shape and amplitude have also been observed by Rickenberg et al. (13) using an immunoassay for cAMP. (2) A detectable rise of the extracellular cAMP concentration begins later than the increase of the intracellular one, but before the latter reaches its peak. This indicates a phase of both high synthesis and release of cAMP. (3) The extracellular cAMP reaches its peak 30 (to 45) seconds later than the internal concentration; this means that cAMP still continues to increase externally when the internal concentration already decreases. This says that the duration of the signalling phase is composed both of the time needed for synthesis of cAMP and for its trans-membrane transport. The half-width of the extracellular spikes is about 100 seconds, which is in agreement with the value of 90 seconds obtained by another technique for the half-width of experimentally induced cAMP-pulses (6), and also agrees with the activity phases of aggregation centers (10). (4) The peak concentration of extracellular cAMP under the experimental conditions is much lower than expected from the internal pulse (fig.2, left ordinate). This is due to dilution of cAMP into the medium as well as to the action of cell-surface phosphodiesterase. (5) If one compares the μmoles of intracellular cAMP which were present before a spike with that in the external peaks (fig.2, right ordinate), one finds a 2 to 3 fold higher quantity in the latter. Thus even if one disregards phosphodiesterase action the conclusion is unavoidable that the majority of the released cAMP is newly synthesized. This result argues against a significant function of any longterm storage form of cAMP, e.g. of cAMP

vesicles, in intercellular signalling. (6) The intracellular cAMP peaks coincide more tightly with the peaks of light scattering changes (top of fig.2) than the extracellular ones. This result suggests that the light scattering changes associated with spontaneous spikes reflect responses of the cells to their internal cAMP pool. (7) In the phase between the spikes the intracellular cAMP concentration is about 3 µM, and the extracellular one under our conditions is approx. 0.1 µM. Under identical conditions but 1 hour after the end of growth, concentrations of 1.4 µM and 0.1 µM, respectively, have been obtained. At this stage no spontaneous periodic activity has been observed. The baseline levels of cAMP within the signalling and non-signalling cells, therefore, differ only by the factor of 2, and the extracellular background levels of both stages are almost identical under given conditions.

Altogether our results suggest a primary role of adenylate-cyclase regulation in oscillatory activity without excluding the involvement of permeability changes in signalling. These results do neither support nor exclude any particular mechanism of cyclase regulation, e.g. the cross activation of cyclase and pyrophosphohydrolase by their products (11). They lend support, however, to the general framework of a theoretical study on cyclase oscillation in which periodic cAMP spikes of similar shape have been obtained (12).

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