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On Induction of Cell Differentiation by Cyclic AMP Pulses in *Dictyostelium discoideum**

B. Wurster

Fakultät für Biologie, Universität Konstanz,
D-7750 Konstanz, Federal Republic of Germany

Abstract. Repeated pulses of cyclic AMP, applied at intervals of 5 min, efficiently induced differentiation in cells of agip 53, a morphogenetic mutant of *Dictyostelium discoideum*, strain Ax-2. In contrast, pulses applied at intervals of 2 min did not induce cell differentiation. To analyze this phenomenon the hydrolysis of cyclic AMP between the pulses as well as the effect of the pulses on the intracellular concentration of cyclic GMP were investigated. Experiments performed in the presence of added cyclic AMP phosphodiesterase revealed that incomplete hydrolysis of cyclic AMP was not the reason for the inefficiency of the pulses applied with a 2-min rhythm. Cyclic AMP pulses applied at intervals of 2 min induced discrete increases of the cyclic GMP concentration. Limited time resolution at the level of cyclic GMP cannot account for the inefficiency of the 2-min pulses.

Key words: cAMP – cGMP – Differentiation – *Dictyostelium*

Upon starvation growth phase cells of *Dictyostelium discoideum* differentiate into aggregation competent cells, which aggregate into multicellular masses (Bonner 1967; Loomis 1975). Cyclic AMP serves as a chemical signal in aggregation. Cells respond chemotactically to cyclic AMP (Konijn et al. 1967) and they are able to produce and release cyclic AMP in an oscillatory manner (Gerisch and Wick 1975). Another chemoattractant for *D. discoideum* cells is folic acid (Pan et al. 1972). Cyclic AMP and folic acid, when supplied as pulses, stimulate cell differentiation to the aggregation competent state (Gerisch et al. 1975; Darmon et al. 1975; Wurster and Schubiger 1977) and induce biochemical oscillations (Wurster and Schubiger 1977). It is conceivable that the reactions regulating cell differentiation are connected with the oscillating reaction system. A reason why pulses induce cell differentiation more efficiently than constant

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attractant concentrations may be adaptation of the pathway that regulates developmental processes (Gerisch et al. 1977).

Cyclic GMP may be involved in the regulation of cell differentiation and oscillations. A pulse of cyclic AMP or folic acid induced a rapid and transient increase of the intracellular cyclic GMP concentration (Mato et al. 1977a, b; Wurster et al. 1977; Wurster and Bumann 1981). Biochemical oscillations are accompanied by periodic changes of the cyclic GMP concentration (Wurster et al. 1977).

The effect of the pulses depends on the rhythm at which they are supplied. At 23° C the cells' inherent rhythm of oscillations is about 8 min (Gerisch and Hess 1974). Folic acid pulses supplied with rhythms between 7 and 11 min efficiently induced oscillations. Folic acid pulses supplied at intervals of 2 min did not induce oscillations (Wurster and Schubiger 1977). Cyclic AMP pulses supplied with rhythms of 5 min (Darmon et al. 1975) or 6.5 min (Gerisch et al. 1975) efficiently induced cell differentiation. Here we report that cyclic AMP pulses supplied at intervals of 2 min do not stimulate cell differentiation. A possible interpretation of the inefficiency of pulses supplied with a rhythm of 2 min is that the cells do not recognize these pulses as discrete signals but as a continuous inefficient one. The reason could be either incomplete hydrolysis of the chemoattractant between two pulses or limited time resolution of the pathway that regulates developmental processes.

Here we attempt to analyze this phenomenon by studying a) the hydrolysis of cyclic AMP between two pulses, catalyzed by cyclic AMP phosphodiesterase (for references see Gerisch and Malchow 1976), and b) the effect of the pulses on the elicitation of concentration increases of cyclic GMP.

Materials and Methods

Chemicals

Adenosine 3′,5′ cyclic phosphate (cyclic AMP) and guanosine 3′,5′ cyclic phosphate (cyclic GMP) were purchased from Boehringer, Mannheim. [8-^{3}H] adenosine 3′,5′ cyclic phosphate, specific activity 30 Ci/mmol was the product of Amersham Buchler, Braunschweig. Cyclic AMP-[^{125}I]-tracer, cyclic GMP [^{125}I] tracer, cyclic AMP antiserum, and cyclic GMP antiserum were obtained from New England Nuclear, Dreieich.

Cyclic nucleotide phosphodiesterase from *Dictyostelium purpureum* was prepared according to published procedures (Tsang and Coukell 1979). This enzyme is only weakly affected by the phosphodiesterase inhibitor produced by *D. discoideum* cells (Gerisch et al. 1972).

Strain and Culture Conditions

Experiments were performed with agip 53, a morphogenetic mutant derived from *D. discoideum,* strain Ax-2 (Darmon et al. 1975). Agip 53 cells normally do

not develop any of the characteristics of aggregation competence unless they are treated with cyclic AMP pulses at appropriate intervals (Darmon et al. 1975). A clone of agip 53 was the generous gift of P. Brachet, Pasteur Institute, Paris. Cells were cultivated on nutrient medium supplemented with 1.8% maltose (Watts and Ashworth, 1970). Streptomycin was added at 20 μg/ml. Cells were harvested at densities of $3-8 \times 10^6$ cells/ml, washed three times in the cold with 17 mM Sørensen phosphate buffer (pH 6.0), adjusted to 1.5×10^7 cells/ml and shaken at 23° C. t_n = time (h) after separation from growth medium.

EDTA-Stable Cell Adhesion

Cells at t_3 were divided into 20 ml portions in 100 ml Erlenmeyer flasks and shaken at 23° C. Cyclic AMP pulses (0.01 ml of 4×10^{-5} M, resulting in a final concentration of 2×10^{-8} M) were applied to the cell suspensions at intervals of 2 or 5 min for periods of 3 h. Cell samples withdrawn from the suspensions were resuspended at a density of 1.5×10^6 cells/ml in 10 mM EDTA in 17 mM Sørensen phosphate buffer pH 6.0. Cells were dissociated by vigorous shaking. Differentiated cells reagglutinate in the presence of 10 mM EDTA (Beug et al. 1973) while undifferentiated cells remain dissociated.

Light Scattering Technique

The responses to cyclic AMP were studied with suspended amoebae at 23° C using an optical technique (Gerisch and Hess 1974) where changes in optical density seem to be due to changes of cell shape (Wurster et al. 1978). At t_3 2 ml of the cell suspension were transferred into a cuvette and agitated by bubbling water-saturated oxygen through the suspension at a flow rate of 28 ml/min. At intervals of 2 min 2 μl of 2×10^{-5} M cyclic AMP (resulting in a final concentration of 2×10^{-8} M) were added. The optical density at 405 nm was measured with a Zeiss PM 6 spectrophotometer.

Assay of Cyclic GMP and Cyclic AMP

Cyclic GMP and cyclic AMP were assayed by means of sensitive radioimmunoassays (Harper and Brooker 1975). Preparation of samples, calibration curves, and controls were as described previously (Wurster et al. 1977).

Assay for Cyclic AMP Hydrolysis

Hydrolysis of cyclic AMP by cell suspensions was determined at 23° C in Sørensen phosphate buffer pH 6.0. Cell suspensions (1.5×10^7 cells/ml) received a pulse of [^{3}H]-cyclic AMP (2×10^{-8} M, final concentrations). After varying time intervals 0.1 ml samples were withdrawn from the suspension, combined

with 0.1 ml of 2 N $HClO_4$, and subsequently neutralized with 3 M K_2CO_3. Cyclic AMP, 5′-AMP and adenosine were separated by chromatography and their proportion determined as previously described (Malchow et al. 1972).

Results

Effect of Cyclic AMP Pulses on Developmental Processes

Cell differentiation from the growth phase to the aggregation competent state is accompanied by changes of biochemical parameters, for instance the number of specific contact sites (known as contact sites A) (Beug et al. 1973) which function in EDTA-stable cell adhesion increase drastically. We used EDTA stable cell adhesion as indicator for cell differentiation. Cells that received pulses of 2×10^{-8} M cyclic AMP at intervals of 5 min developed EDTA-resistant adhesive properties as previously reported (Darmon et al. 1975). In contrast, cells that were supplied with cyclic AMP pulses at intervals of 2 min were not able to form EDTA-stable aggregates (data not shown).

Degree of Cyclic AMP Hydrolysis Between Two Pulses

We determined the half-life of cyclic AMP in cell suspensions before and after treatment with cyclic AMP pulses. Unpulsed cell suspensions at t_3 catalyzed the hydrolysis of cyclic AMP at a half-life of 90 s (± 30 s). From this value the degree of hydrolysis of cyclic AMP between the first two pulses was calculated as 62% (± 12%) hydrolysis within 2 min. Cyclic AMP pulses supplied at intervals of 2 min caused increases of the activity of extracellular cyclic AMP phosphodiesterase (data not shown). Cell suspensions at t_6, which were pulsed from t_3 to t_6 at intervals of 2 min, hydrolyzed cyclic AMP at a half life of 7 s (± 2 s).

The incomplete hydrolysis of cyclic AMP within 2 min at the beginning of the pulse treatment prompted us to perform experiments in the presence of added cyclic AMP phosphodiesterase. An activity of 80 nmol $\times$ min^{-1} $\times$ ml^{-1} was added to cell suspensions at t_3 resulting in a half-life time for cyclic AMP hydrolysis of 5 s. When these cell suspensions were supplied with cyclic AMP pulses at intervals of 2 min for 3 h they did not develop the capacity to form EDTA-resistant adhesive properties (data not shown).

Effect of Cyclic AMP Pulses on the Concentration of Cyclic GMP and on a Cellular Reaction

We examined the concentration of cyclic GMP in response to cyclic AMP pulses, applied at intervals of 2 min. In addition, a complex reaction of the cells was recorded by measuring light scattering throughout the experiments. The experiment shown in Fig. 1 was performed in the absence of added cyclic AMP phosphodiesterase. The 1st pulse caused a decrease in optical density which still

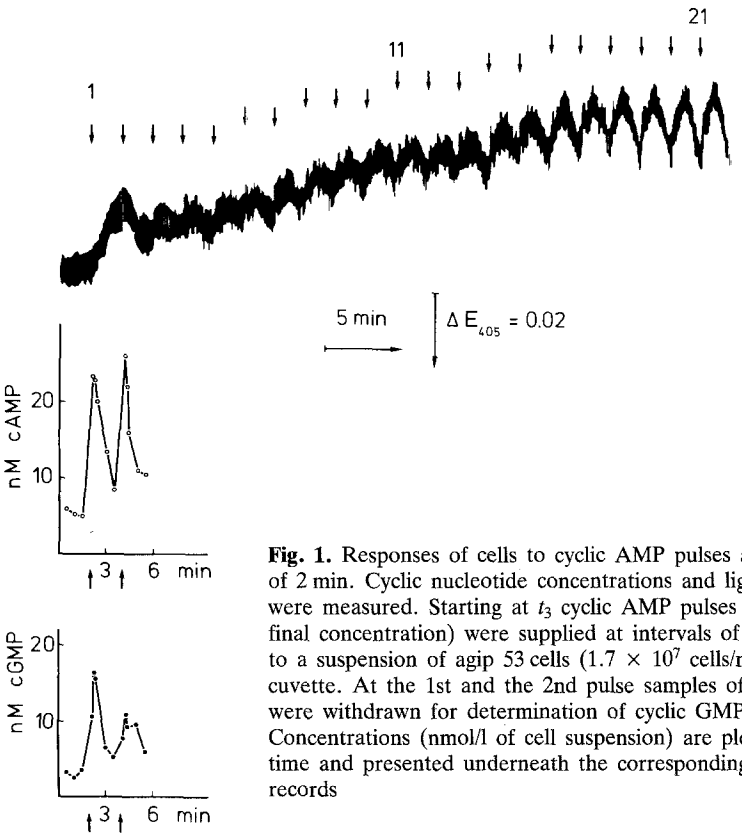

Fig. 1. Responses of cells to cyclic AMP pulses applied at intervals of 2 min. Cyclic nucleotide concentrations and light scattering were measured. Starting at t_3 cyclic AMP pulses (2×10^{-8} M, final concentration) were supplied at intervals of 2 min (arrows) to a suspension of agip 53 cells (1.7×10^7 cells/ml) in an optical cuvette. At the 1st and the 2nd pulse samples of 30 µl volume were withdrawn for determination of cyclic GMP and cyclic AMP. Concentrations (nmol/l of cell suspension) are plotted against the time and presented underneath the corresponding light scattering records

persisted by the time the 2nd pulse was supplied. The 2nd pulse did not elicit a discrete reaction. The resolution of the reactions became better with increasing number of pulses until after about 20 pulses optimal resolution was attained. At the 1st and the 2nd pulse samples were withdrawn for determination of cyclic GMP and cyclic AMP. The addition of a cyclic AMP pulse resulted in an instantaneous rise of the cyclic AMP concentration in the cell suspension and subsequent hydrolysis of cyclic AMP. The 1st pulse induced a rapid and transient 5-fold increase, and the 2nd pulse caused a 2- to 3-fold increase of the cyclic GMP concentration.

Similar experiments were performed in the presence of added cyclic AMP phosphodiesterase (80 nmol $\times$ min^{-1} $\times$ ml^{-1}). Light scattering reactions in response to cyclic AMP pulses were clearly resolved and did not change significantly from the 1st pulse to the 91st pulse (Fig. 2). The pattern was similar to that observed after the initial series of pulses in the absence of added cyclic AMP phosphodiesterase (Fig. 1).

Samples were withdrawn at the 1st, 2nd, 90th, and 91st pulse. The cyclic AMP added in the pulses was rapidly hydrolyzed (Fig. 2). The cyclic GMP concentration after the 1st, 2nd, 90th, and 91st pulse increased by factors of 9, 4, 6, and 9, respectively.

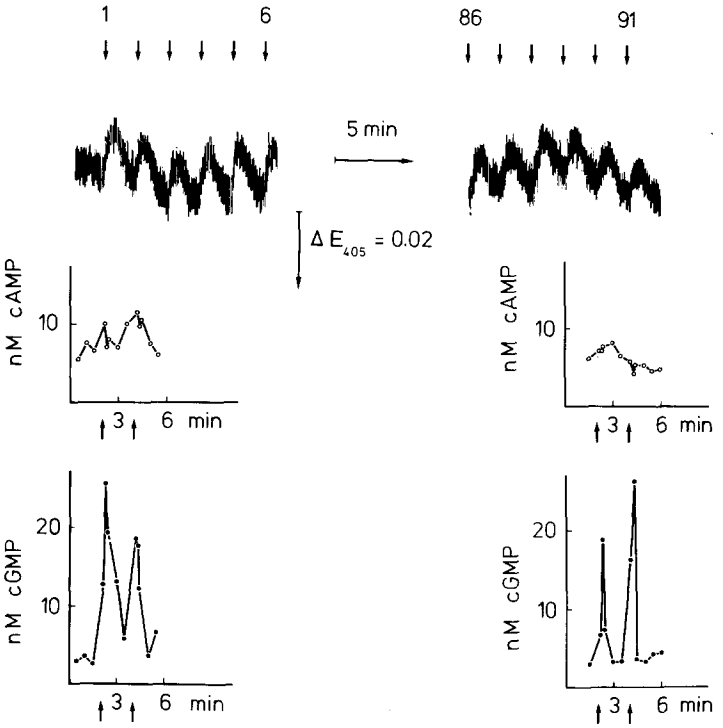

Fig. 2. Effect of added cyclic AMP phosphodiesterase on the responses of cells to cyclic AMP pulses. Cyclic nucleotide concentrations and light scattering were measured. At t_3 to a suspension of agip 53 cells (1.7×10^7 cells/ml) cyclic AMP phosphodiesterase (80 nmol $\times$ min^{-1} $\times$ ml^{-1}) was added. Subsequently the cells received cyclic AMP pulses (2×10^{-8} M, final concentration) at intervals of 2 min (arrows). At the 1st, 2nd, 90th, and 91st pulse samples of 30 µl volume were withdrawn for determination of cyclic GMP and cyclic AMP. Concentrations (nmol/l of cell suspension) are plotted against the time and presented underneath the corresponding light scattering records

Discussion

One possible interpretation of the inefficiency to induce cell differentiation with cyclic AMP pulses applied at intervals of 2 min was that the cells do not recognize these pulses as discrete signals, but as a continuous inefficient one. Results reported here show that incomplete hydrolysis of cyclic AMP is not the reason for the inefficiency of these pulses. There remains the question whether limited time resolution of the signal processing system is the reason for the inefficiency of the 2-min rhythm. Our results show that pulses applied at intervals of 2 min were resolved as discrete signals at the level of cyclic GMP. Assuming cyclic GMP is part of the pathway that regulates developmental processes, limited time resolution may occur in a reaction succeeding cyclic GMP.

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