CYCLIC GMP IN *DICTYOSTELIUM DISCOIDEUM*

Oscillations and pulses in response to folic acid and cyclic AMP signals

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1. Introduction

Cells of *Dictyostelium discoideum* respond chemotactically to cyclic AMP [1] and to folic acid [3]. Cyclic AMP is a most efficient attractant for cells of the aggregation phase [2], folic acid is more effective with preaggregation cells [4]. Both cyclic AMP and folic acid stimulate also cell development from the preaggregation phase to the aggregation competent state [5–8]. During this process the cells acquire the capacity to synthesize cyclic AMP periodically, and to release it into the extracellular space in form of pulses [9]. The administration of cyclic AMP or folic acid pulses accelerates the onset of sustained oscillations [5,8].

The cyclic-AMP induced responses are known to be mediated by cell-surface receptors [10–12]. The intermediate steps in signal processing from the receptors to the various intracellular targets are unknown. In the present communication we show that upon stimulation of early preaggregation cells with folic acid a rapid increase of the cyclic GMP concentration is induced. A biphasic increase of cyclic GMP is observed in late preaggregation cells stimulated by cyclic AMP. The second cyclic GMP peak is succeeded by a cyclic AMP peak known to be based on the activation of adenylate cyclase [13]. Free-running oscillations of cyclic GMP were observed together with the periodic formation of cyclic AMP pulses, and the cyclic GMP peaks seemed to occur slightly in advance of the cyclic AMP peaks.

2. Material and methods

Folic acid was purchased from Serva, Heidelberg; cyclic AMP, cyclic GMP, and beef heart phosphodiesterase (EC 3.1.4.17) were obtained from Boehringer, Mannheim. Cyclic AMP- and cyclic GMP-antibodies as well as ^{125}I-labelled cyclic AMP and cyclic GMP derivatives [14] were kindly provided by Dr H. L. Cailla, Marseille.

Cells of *D. discoideum*, strain Ax-2, clone 206, were cultivated on nutrient medium supplemented with 1.8% maltose [15]. The amoebae were harvested at densities of $3–7 \times 10^6$ cells/ml, washed three times in the cold with 17 mM Soerensen phosphate buffer, pH 6.0, adjusted to 1×10^7 cells/ml, and shaken at 23°C. The time of resuspension was taken as the beginning of cell development to the aggregation competent state which was reached after 5–7 h.

At various stages of development the shaken suspension was centrifuged and the cells resuspended in the buffer at a concentration of 5×10^7/ml. The suspension was agitated in an optical cuvette by bubbling water-saturated oxygen at a rate of 24 ml/min through the suspension [5].

For the determination of cyclic nucleotides, 40 µl samples were withdrawn from the cuvette, immediately mixed with perchloric acid (1 N final concentration) and neutralized with K_2CO_3. The sensitive radioimmunoassay of Cailla et al. [14] was used as modified by Harper and Brooker [16].

The specificity of the assay was tested by adding a

40-fold excess of cyclic AMP to aliquots assayed for cyclic GMP, and vice versa. No cross-reaction was observed. The assay is affected by unspecific material present in extracts of *D. discoideum* cells [17]. Therefore, in each experiment a perchloric acid extract was treated with beef heart phosphodiesterase (0.1 units/ml) for 1 h at room temperature and heated in a boiling water bath. Calibration curves for the immunoassay were obtained from cyclic AMP and cyclic GMP standards to which the digest was added in amounts equivalent to the sample used. In addition, samples in which peak concentrations of cyclic GMP and cyclic AMP had been found, were treated with phosphodiesterase as above to establish hydrolysis of the antibody-binding material.

No distinction was made between intra- and extracellular cyclic nucleotides. Previous results have shown that the cyclic AMP is rapidly hydrolysed in the extracellular space [10] and that also cyclic GMP is hydrolysed by the *D. discoideum* phosphodiesterases [18]. It was reasonable therefore to calculate the cyclic nucleotide concentration/liter densely packed cells. The volume of the cell-sediment was 4% of the total volume; therefore the values given have to be divided by 25 in order to obtain the average concentrations in the cell-suspensions.

3. Results

3.1. *Oscillations of cyclic GMP*

Together with sustained oscillations of light scattering, periodic changes of the cyclic GMP concentrations were observed. Figure 1 shows that the cyclic GMP peaks were accompanied, probably with a slight delay, by cyclic AMP peaks of 5- to 10-fold higher amplitude. At 23°C the temporal resolution was however not sufficient to establish the delay of the cyclic AMP peaks unequivocally. Therefore, the following experiments were carried out at 10°C.

3.2. *Increases of cyclic GMP induced by cyclic AMP*

As shown in fig.2 a cyclic AMP pulse elicited a biphasic light scattering change in late preaggregation cells [5]. At 10°C these responses were about 3-fold slower than at 23°C. The increase of cyclic GMP was resolved into two phases, with peaks after 25 s and 3 min. The first measurement after stimulation

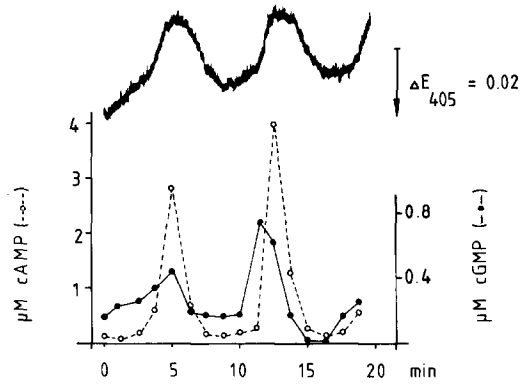

Fig.1. Oscillations of cyclic GMP and cyclic AMP. Autonomous oscillations were induced by a series of 11 folic acid pulses applied in intervals of 8 min, starting 2 h after separation from the growth medium [8]. Light scattering changes were used as an indicator of the oscillations. Cyclic GMP and cyclic AMP were calculated/liter cell-sediment.

indicates that the cyclic GMP started to rise within the first 9 s. The increase of cyclic AMP became detectable not earlier than 30 s after stimulation, and reached its maximum at 3.5 min. The cyclic AMP increase was not resolved into two peaks.

In the experiment shown the cyclic GMP increased in the first peak from about 0.05–0.65 μmol/liter cell-sediment, in another experiment from 0.12–1.0.

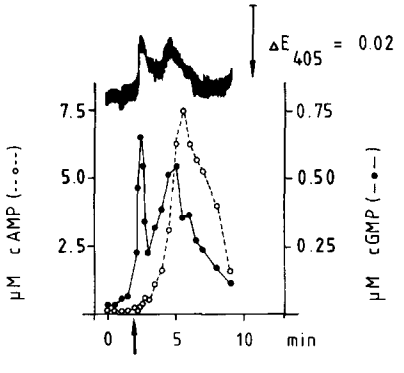

Fig.2. Increases of cyclic GMP and cyclic AMP induced by cyclic AMP. To a suspension of late preaggregation cells (4 h after separation from the growth medium) a pulse of cyclic AMP was applied (5×10^{-9} M final concentration), as indicated by the arrow. (– • –) Cyclic GMP; (– – o – –) Cyclic AMP/liter cell sediment. On top, concomitant light scattering changes are shown.

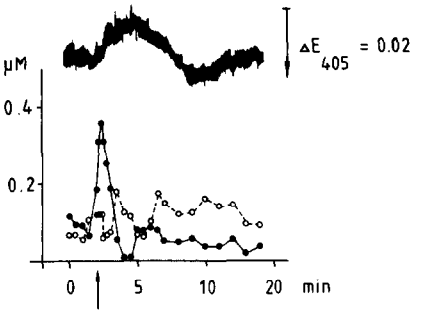

Fig.3. Cyclic GMP increase induced by folic acid. Early pre-aggregation cells (2 h after separation from the growth medium) were stimulated by folic acid (2×10^{-7} M final concentration) as indicated by the arrow. (– • –) Cyclic GMP; (– – ○ – –). Cyclic AMP calculated/liter cell-sediment. Light scattering changes are shown on top.

The cyclic AMP increased about 100-fold from 0.07–7.5 μmol.

3.3. *Cyclic GMP increase induced by folic acid*

Figure 3 shows that the addition of folic acid to preaggregation cells at 10°C caused a rapid increase of the cyclic GMP concentration. This rise was already observed in the sample taken 9 s after stimulation, and the maximal concentration was reached after 25 s. This sharp maximum preceded the peak of the light scattering change.

Calculated/liter cell-sediment, the cyclic GMP increased from about 0.07–0.36 μmol in the experiment shown, and from 0.1–0.62 in a second experiment. In the experiment of fig.3 the cyclic AMP concentration remained constant within the limits of 0.1 ± 0.06 μmol, in contrast to the 5-fold increase of cyclic GMP.

The absence of a significant cyclic-AMP increase after folic acid stimulation can not be generalized. In an advanced stage of cell development such an increase has been observed (Wurster and Schubiger, in preparation).

4. Discussion

The results reported in the present paper are compatible with a role of cyclic GMP in chemotaxis, receptor-mediated adenylate cyclase activation, autonomous oscillation and cell differentiation.

At about 23°C the beginning of a chemotactic response, as reflected by the extension of pseudopods towards a cyclic-AMP gradient, was observed 5 s after cyclic AMP application [19]. At 10°C the cyclic GMP increase within 9 s after administration of either folic acid or cyclic AMP. Taking into account a factor of about 3 for the temperature-dependence of the responses, the increase of cyclic GMP most likely begins before 3 s after stimulation.

A second rise of cyclic GMP in advance of a cyclic AMP pulse (fig.2) would be in accord with a role of cyclic GMP in the generation of cyclic AMP signals. The cyclic GMP changes were smaller than those of cyclic AMP. This suggests a cyclic GMP/cyclic AMP cascade and a function of guanylate cyclase in signal amplification.

In the case of fig.3 no significant increase of cyclic AMP in response to folic acid was observed. In the developmental stage used, folic acid has already a stimulating effect on cell development [8]. This poses the question whether cyclic GMP functions independently of cyclic AMP as an intracellular mediator of cell differentiation.

It is tempting to speculate that specific intracellular targets should exist for the recognition of either cyclic GMP or cyclic AMP changes. Cyclic nucleotide dependent proteinkinases could play this role.

Acknowledgment

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