AMPLIFICATION OF CYCLIC-AMP SIGNALS IN AGGREGATING CELLS OF *DICTYOSTELIUM DISCOIDEUM*

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1. Introduction

Aggregating cells of the slime mold, *Dictyostelium discoideum*, respond to cyclic AMP by chemotactic orientation [1,2] and by propagating chemotactic signals in the form of waves from one cell to the next [3,6]. It has been proposed that each cell acts as a biochemical signal amplifier [3–8] so that cyclic AMP would play the role of a transmitter in a relay system. In order to investigate the amplifier system quantitatively, methods have been developed to measure signal generation, cyclic-AMP binding to receptors as well as cellular responses to cyclic AMP in stirred cell suspensions [9–11]. Under these conditions evidence for signal amplification has been obtained [11–13] using the protein-binding assay for cyclic-AMP [14]. In the present paper we report results obtained by an independent method for measuring cyclic-AMP stimulated cyclic-AMP release: prelabelling of cells with [^{3}H]adenine and chromatographic separation of the labelled compounds from the extracellular fluid after stimulation of the cells by unlabelled cyclic AMP.

2. Methods

Cells of the axenic *D. discoideum* strain Ax-2 were cultivated on a gyratory shaker in growth medium containing 1.8% maltose [15] up to a final density of 5×10^6 cells/ml, washed and resuspended in 17 mM phosphate buffer pH 6.0 to induce differentiation to aggregation-competence [16]. If not stated otherwise, the cells were harvested 5 to 7 hr later for labelling with 2-[^{3}H]adenine (Amersham). Adenine uptake was measured by shaking 5×10^7 cells at 23°C in 250 μl phosphate buffer containing 0.2 to 0.8 μCi [^{3}H]adenine. At intervals of 5 min cells from 50 μl samples were washed 5 times with cold phosphate buffer, the cell sediments extracted with 0.5 M perchloric acid, the extracts neutralized and counted in a triton-toluene-scintillator. The rate of uptake was a linear function of both time and adenine concentration up to 15 min and 15 μM adenine, and rates were similar both in cells measured shortly after washing free of nutrient and 8 hr later (fig.1).

To determine the spectrum and relative activity of labelled compounds, 100 μl cell suspension containing 2×10^7 cells were incubated with 30 μCi [^{3}H]adenine

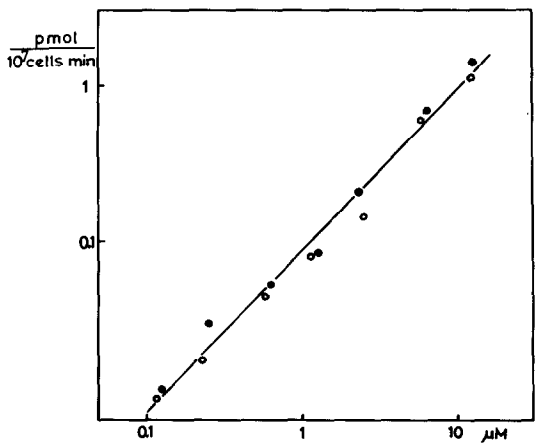

Fig.1. Initial rates of uptake of [^{3}H]adenine by cells at 2 hr and 8 hr after removal from growth medium: (o–o–o) 8-hr cells; (•–•–•) 2-hr cells.

(specific activity 17 Ci/mmol) for 30 min, the cells washed as above and resuspended in the original volume of phosphate buffer. After shaking for 10 min at 23°C, perchloric acid was added to the total suspension and, after neutralization, 10 μl of the extract chromatographed on PEI-cellulose [17].

For the separation of all compounds by two-dimensional thin-layer chromatography, two sheets were required. In the first dimension both plates were developed first with distilled water, second with 0.5 M lithium chloride up to a length of 80% of the water run. In the second dimension the first sheet was developed with 4 M lithium chloride/1 *N*-acetic acid (3:7) for separation of the nucleoside di- and triphosphates; the second sheet with 0.5 M sodium formate buffer pH 3.4 for separation of the other compounds. Authentic references were added before separation. For counting in triton-toluene-scintillator the spots were eluted using 0.7 M magnesium chloride in 0.02 M Tris–HCl buffer [17].

For determining cyclic-AMP release 4×10^8 cells per ml in phosphate buffer were labelled for 1 hr at room temperature with 100 to 150 μCi [^{3}H]adenine per ml, specific activity 15.5 Ci/mmol. After 6 times washing in cold phosphate buffer, the cells were adjusted to 2×10^8/ml and preincubated under oxygen bubbling for 10 min at room temperature before stimulation by pulses of 6×10^{-8} M cyclic AMP.

In microcentrifuge tubes, 300 μl silicone oil (AR 20:AR 200 1:1 v/v; Wacker Chemie, München, Germany) was layered on top of 30 μl 15 mM cyclic AMP. At intervals before and after stimulation, 30 μl samples of the cell suspension were pipetted on top of the silicone and centrifuged in a Beckman microfuge. The cells instantaneously sedimented to the bottom of the silicone layer and simultaneously the cyclic AMP solution came on top of it where it mixed into the extracellular fluid preventing hydrolysis of the released [^{3}H]cyclic AMP. This upper phase was heated 5 min at 100°C, chromatographed on PEI-cellulose in the first dimension as described above, and counted. The identity of the separated compound as cyclic-AMP was confirmed by incubation with beef-heart cyclic-AMP phosphodiesterase (0.15 units/mg, Boehringer Mannheim) for 2 hr at 25°C and pH 7.5. ATP was determined by the luciferase assay [18] using the bioluminescence equipment and Fisons injector of Skan AG, Basel, Switzerland.

3. Results and discussion

3.1. *Spectrum of labelled compounds*

Cells were labelled with [^{3}H]adenine either 1 or 7 hr after replacing the nutrient medium by phosphate buffer. The distribution of the label was similar in cells of these two developmental stages, and also similar to data reported for the 'slug' stage [19], with the significant exception that guanosine nucleotides were higher labelled in the 7-hr stage than before (table 1). This result is relevant because GTP is an activator of adenylcyclase in eucaryotic cells [20], and in *D. discoideum* intra- and extracellular cyclic-AMP levels increase within a period of 8 hr after the end of growth [21]. Several developmental activities ascribed to cyclic-AMP increase or start within the same period [1,8,9].

3.2. *Triggering of cyclic-AMP release*

Cells prelabelled with [^{3}H]adenine at the 5 to 6-hr stage were stimulated by cyclic-AMP pulses with an amplitude of 6×10^{-8} M (this would be the final concentration in the cell suspension if no cyclic AMP were hydrolysed within the short period required for injection by hand). Following injection, a rise of the label in the extracellular cyclic-AMP fraction was obtained, with a peak after about 90 sec, and a half-width of 90 sec (fig.2). Sensitivity to cyclic-AMP phosphodiesterase proves that the evoked release is of cyclic AMP.

Table 1
Distribution of the 2-[^{3}H]adenine label within the perchloric-acid soluble fraction as per cent of the total label

	1-hr cells	7-hr cells
ATP	63.7	42.0
ADP	15.4	16.7
AMP	4.1	4.5
cAMP	0.1	0.2
adenosine	2.4	2.3
adenine	0.8	1.9
GTP + GDP	9.4	27.7
GMP	0.4	1.2
cGMP	⩽0.2	⩽0.1
guanosine	3.1	3.2
IMP	0.3	0.2

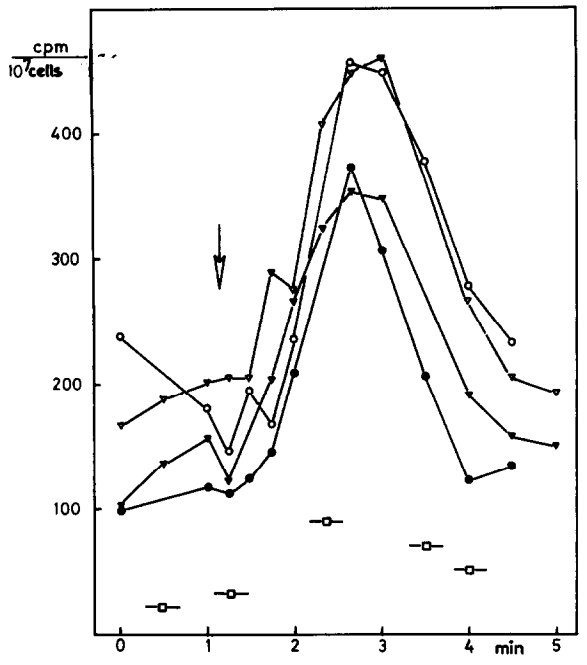

Fig.2. Changes of the extracellular cyclic-AMP label in a cell suspension which was stimulated 4 times at intervals of 8 min by pulses of 6×10^{-8} M cyclic AMP: (●–●–●) 1st, (▼–▼–▼) 2nd, (○–○–○) 3rd, (▽–▽–▽) 4th stimulation. (–□–) label left in cyclic-AMP spots after phosphodiesterase incubation of a mixture of the four runs. Arrow: injection of 6×10^{-8} M cyclic AMP.

The triggered responses shown in fig.2 agree quantitatively with respect to overall shape and half-width with the signalling periods of aggregation centers (fig.12 of ref. [7]). They are supposed to provide a reliable picture of the shape of the signalling curve because the cell concentration chosen was sufficiently high (15 to 20% v/v) for rapid hydrolysis of the released cyclic AMP by cell-surface phosphodiesterase [10,16]. The length of signalling is of an order to suggest that the minimal period of spontaneous pulsing, which was found by several groups to be about 2.5 min [7,22,23] is just the minimal interval at which consecutive signals do not overlap.

We must mention, however, that such uniform responses have not always been obtained. Particularly in 9-hr cells, responses of varying shape and amplitude have been found both using the protein-binding assay [14] and the adenine-labelling technique. Often a fast response with a peak within the first half minute was followed by a second peak 2-3 min after stimulation [12]. The fast peak possibly corresponds to the shoulders indicated in two of the responses shown in fig.2. Because cells at the 9-hr stage are able to oscillate spontaneously [9], we suppose that their output results from the interplay of the experimental rectangular cyclic-AMP stimulus with the cellular oscillator, and depends on its phase.

3.3. *Estimates of amplification factors*

In a series of seven experiments using the protein-binding assay, a mean increase of the extracellular cyclic-AMP concentration by 5×10^{-7} M was evoked within one minute by pulses of 5×10^{-8} M cyclic AMP, indicating signal amplification by a factor of 10 in terms of output/input concentration changes [11–13]. Under the same conditions, spontaneous oscillations of the extracellular cyclic-AMP level with an amplitude of 1 μM have been measured [12,13] providing evidence for the autonomous generation of cyclic-AMP pulses in the same order of magnitude as the experimentally induced ones. The cell concentration in these experiments was 2×10^8/ml. Using realistic values for the activity and the kinetics of cell-surface phosphodiesterase [16,24], and neglecting extracellular phosphodiesterase in the suspensions of freshly washed cells, a steady-state approximation yields at least 6×10^6 as the total number of cyclic-AMP molecules released per cell in response to a stimulus. Since the latter corresponds to 1.5×10^5 molecules per cell, the output/input ratio expressed as number of molecules is in the order of 40. These are certainly not maximal values because part of the molecules will be hydrolysed by cell-surface phosphodiesterase before they bind to receptors, and also because the stimulating dose used does not saturate the cyclic-AMP binding sites at the cell surface [10] or the cellular responses recorded by light scattering measurements [9].

In accord with previous determinations [25], the cellular ATP level was found to be 1.5 mM under our conditions. On the basis of this value and the distribution of label shown in table 1, the specific activity of ATP was estimated. Assuming that cyclic AMP had the same specific activity as its precursor, amplification factors in the same order as those obtained by the protein-binding method were

assessed: as a mean from 8 experiments in which 2×10^8 cells/ml were stimulated by 6×10^{-8} M cyclic AMP, an increase of extracellular concentration of labelled cyclic AMP by $36 \pm 11 \times 10^{-8}$ M was calculated, which corresponds to a mean amplification factor of 6 in terms of output/input concentration changes. In conclusion, the results both of the protein-binding assay and of adenine-labelling demonstrate that the cells are able to amplify cyclic-AMP pulses, as is necessary for the cyclic-AMP acting as a transmitter in the morphogenetic interactions of aggregating *D. discoideum* cells.

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