CYCLIC AMP AS A CELL SURFACE ACTIVATING AGENT IN *DICTYOSTELIUM DISCOIDEUM**

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1. Introduction

Cyclic AMP (cAMP) generally functions as a regulator of biological processes within the cell (see ref. [1]). In some species of *Dictyostelium* this cyclic nucleotide is essential for extracellular communication (see ref. [2]).

Amoebae of *Dictyostelium discoideum* feed on bacteria. When the food has been exhausted the amoebae secrete cyclic AMP in sufficiently large amounts to attract each other and to form aggregates. At the same time the amoebae also become much more sensitive to the chemotactic effect of cAMP [3].

The role of cyclic AMP seems to be analogous to that played by hormones in higher organisms [2]. The mechanism by which cAMP mediates the chemotactic effect is unknown.

To study the mechanism of chemotaxis it is essential to know whether cAMP acts on the cell surface or penetrates through the cell membrane to function intracellularly. Our results using ^{32}P- and ^{3}H-labeled cAMP indicate that cAMP acts on a component of the cell surface and does not have to penetrate through the cell membrane to exert its chemotactic effect.

2. Materials and methods

The amoebae of *D. discoideum*, NC-4(H), were grown in association with *Escherichia coli*, 281, on a glucose–peptone medium (Glucose, 10 g; Peptone, 10 g; $Na_2HPO_4 \cdot 12\,H_2O$, 1 g; KH_2PO_4, 1.5 g; agar, 15 g; distilled water, 1 l.). The amoebae were harvested in a diluted Bonner's salt solution and washed several times to remove the bacteria [4]. The final concentration of the amoebae was $7–8 \times 10^7$ cells/ml. To 5 ml of amoebal suspension was added 40 µl of [^{3}H] cAMP (1 m Ci/ml, 25 Ci/mmole; Amersham Searle, Chicago, I11.) and a quantity of [^{32}P] cAMP to produce matching counts (0.25 mCi/ml, 1.26 Ci/mmole at time of shipment, International Chemical Nuclear Corp., Irvine, Calif.). The sample shown in table 1 was run 23 days after shipment, so that 40 µl contained 3.6 µCi of [^{32}P] cAMP, giving an expected $\frac{1}{50} \times 3.6 \times 2.2 \times 10^6 = 158{,}400$ dpm for the supernatant if all cAMP remained there. Forty microliters of [^{3}H] cAMP should produce 1.8×10^6 dpm. Samples of 1 ml were

Table 1
The activities in 0.1 ml fractions 20 min after the addition of ^{3}H- and ^{32}P-labeled cAMP to a suspension of 7.5×10^7 amoebae per ml

	Supernatant		Amoebae	
	^{32}P (dpm)	^{3}H (dpm)	^{32}P (dpm)	^{32}H (dpm)
Adenine	2320	43,500	25	590
Adenosine	4400	124,000	6	70
cAMP	5530	100,000	48	550
AMP	66,700	1,055,000	37	400
ADP	14,550	330,000	13	600
P + ATP	27,600	120,000	51	540
Total	121,100	1,772,500	180	2830

* The experiments were carried out in the Hubrecht Laboratory, Utrecht, The Netherlands.

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withdrawn after 1, 10 and 20 min. The samples were cooled to 4°C and centrifuged at 100 *g* for 5 min. The supernatant was frozen immediately and dried under vacuum. The amoebae were washed three times after which 1 ml of 7% perchloric acid was added to the pellet. The amoebae were sonicated for 1.5 min. The broken cell suspension was neutralized with 1 N KOH and allowed to precipitate at 4°C for 30 min. After centrifugation the supernatant was freeze-dried. 1 ml of a 50% methanol solution was added to the freeze-dried residue of the amoebae and 0.1 ml of 50% methanol to the freeze-dried supernatant. Adenine, adenosine, ADP, ATP and cold cAMP were added to the dissolved amoebal residue and to the supernatant.

Twenty microliters of the amoebal extract and 10 μl of the supernatant fraction were deposited on cellulose thin-layer chromatography plates [5]. The solvent consisted of isobutyric acid, ammonium hydroxide and distilled water (64:3:23 v/v). The location of the chromatographed nucleotides was spotted with ultraviolet light. The spots were scraped off and the activity of ^{32}P and ^{3}H was counted on a Nuclear Chicago liquid scintillation counter. Efficiencies were calculated by a channels' ratio method (modification of [6]).

Autoradiography was carried out by exposing amoebae to [^{3}H]cAMP. Afterwards the amoebae were washed three times and either placed directly on gelatin-coated slides or fixed in glutaraldehyde, embedded in Epon and placed on slides after sectioning.

The slides were covered with Kodak AR 10 stripping film. After exposure during 1–2 weeks the slides were developed in Kodak D 19 for 4 min.

3. Results and discussion

Autoradiography of whole cells and sectioned cells resulted in a count of grains in the cells which did not exceed the count of grains in the background. Similarly, cAMP and adenine-containing derivatives in the amoebal fraction showed a very low radioactivity, even after the cells had been exposed to [^{32}P]cAMP and [^{3}H]cAMP for a period of 20 min. The experiment was repeated 6 times and gave similar results. The counts of one experiment are shown in table 1.

Only 0.1–0.2% of the total activity was associated with the amoebae. Part of this activity may have been due to contamination with labeled cAMP, which remained on the cell surface after the amoebae were washed. Both these techniques, autoradiography and liquid scintillation counting, show no association of the amoebae with appreciable amounts of labeled cAMP or its derivatives.

We cannot exclude the possibility that very small amounts of cAMP enter the cell. Since amoebae, however, respond chemotactically to extremely low concentrations of cAMP [2], it becomes unlikely that sufficient molecules could penetrate through the membrane to activate a component within the cell that would initiate the directed movement. Another possibility is an immediate hydrolysis of the cAMP when it enters the cell. If that would be the case one would expect a high radioactivity in break-down products of cAMP inside the amoebae. The activity of other labeled compounds within the cells is also low if compared with the radioactivity in the supernatant. This suggests that amoebae are activated by cAMP, in an analogous way to target cells by hormones: through a stimulation of the cell membrane.

The activity of the labeled cAMP decreased markedly during the first few minutes (fig. 1). At the same time labeled AMP shows a sharp increase in activity. Although the first sample was taken after one min, the counts were marked in the curve after 5 min to allow time for handling the sample until freeze-drying. The activities at 0 min are those of labeled cAMP solutions without amoebae. Six replicates showed the same rapid conversion of cAMP into AMP. The radioactivity accumulates later in the inorganic phosphorus and adenine-containing derivatives.

When the amoebae had already started to aggregate, the conversion of cAMP to AMP was much slower. Whether the reduced breakdown of cAMP has been caused by the presence of a phosphodiesterase inhibitor [7] or by the reduced number of single amoebae in the medium, or by both, is not known.

When labeled cAMP was added to a supernatant in which amoebae had been suspended for only 5 min, a small percentage of the cAMP was broken down. In a supernatant in which the amoebae had been suspended for 12 hr, the labeled cAMP was converted to AMP at a similar rate as shown in fig. 1. Apparently some time is required to build up an extracellular concentration of cyclic nucleotide phosphodiesterase to inactivate the cAMP in the medium effectively.

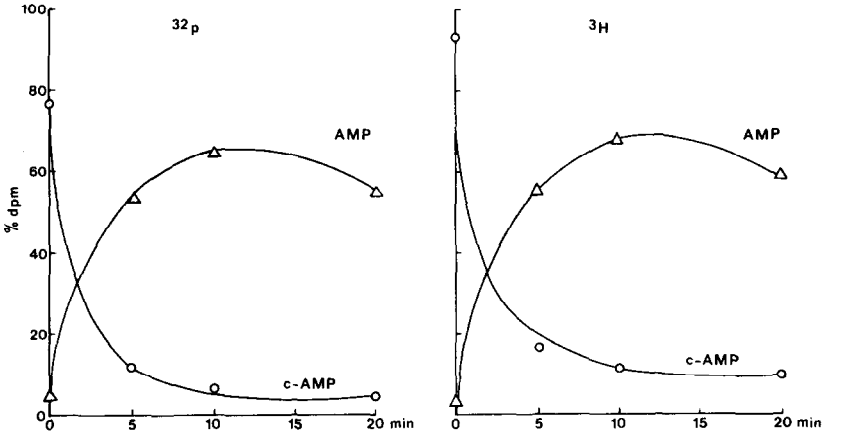

Fig. 1. At time zero [^{32}P]cAMP and [^{3}H]cAMP were added to a suspension of amoebae.

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