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Identification of Adenosine-3',5'-Monophosphate as the Bacterial Attractant for Myxamoebae of *Dictyostelium discoideum*

T. M. KONIJN, J. G. C. VAN DE MEENE, Y. Y. CHANG, D. S. BARKLEY, AND J. T. BONNER

Hubrecht Laboratory and the University of Utrecht, Utrecht, the Netherlands, and Department of Biology, Princeton University, Princeton, New Jersey 08540

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Adenosine-3',5'-cyclic monophosphate was shown to be the compound found in *Escherichia coli* responsible for the attraction of the amoebae of the cellular slime mold *Dictyostelium discoideum*. A number of other nucleotides were tested and the following were active: tubercidin-3',5'-cyclic monophosphate, N^6-2'-*O*-dibutyryl-adenosine-3',5'-cyclic monophosphate, 5'-methylene adenosine-3',5'-cyclic monophosphonate, guanosine-3',5'-cyclic monophosphate, uridine-3',5'-cyclic monophosphate, cytidine-3',5'-cyclic monophosphate, inosine-3',5'-cyclic monophosphate, and thymidine-3',5'-cyclic monophosphate. They were less active than adenosine-3',5'-cyclic monophosphate. It is suggested that cyclic adenosine monophosphate secreted by the bacteria is used by the amoebae as a means of sensing and orienting towards food.

Adenosine 3',5'-cyclic monophosphate (3',5'-cyclic AMP) has been recognized for several years as being one link in a number of hormone-mediated communication systems in mammals. Primarily through the work of Sutherland and his associates, the role of cyclic AMP in the mechanism of action of epinephrine and other hormones is firmly established (10).

Recently we showed that this substance is also important in mediating chemotaxis in the cellular slime molds (7, 8). If cyclic AMP is placed near a drop of sensitive amoebae, the amoebae will orient towards the attracting substance.

It was previously shown that water-wash extracts of *Escherichia coli* are capable of attracting amoebae (5). It is also known (9) that *E. coli* produces 3',5'-cyclic AMP. In this paper we present evidence that 3',5'-cyclic AMP is the compound in the bacterial extracts that attracts the amoebae of the cellular slime mold *Dictyostelium discoideum*. We also have evidence that other nucleotides will exert an attraction on the amoebae.

MATERIALS AND METHODS

Extraction procedure. *E. coli* B/r was grown on a tryptone (0.5%)-yeast extract (0.5%)-glucose (0.1%)-K_2HPO_4 (0.1%)-agar medium in 15 trays (39 by 39 cm) at 30 C for 2 days. The bacteria were then washed off the trays with 1 liter of distilled water and immediately precipitated by centrifugation at 22 C.

The clear water wash of the cells was concentrated to 10 ml in a vacuum evaporator at 50 C and then centrifuged at 22 C to remove the precipitated solids. Such a preparation showed high activity in the attraction assay and remained stable at room temperature.

Assay. Amoebae were grown with *E. coli* 281 on buffered glucose-peptone-agar (1). The cells were harvested after 40 hr of incubation at 21 ± 1 C, and the excess bacteria were washed free of the amoebae by two or three successive centrifugations in Bonner's salt solution (1) diluted 100 times. After suspension in an undiluted salt solution, the concentration of amoebae was adjusted so that droplets of 0.6 mm in diameter on hydrophobic agar contained about 1,000 cells each (for further details of the method, *see* 4, 6).

The agar was repeatedly washed with distilled water, partially dried, and suspended in Bonner's salt solution. By carefully controlling the concentration of the agar, it is possible to have a surface of a critical rigidity: stiff enough to keep the cells within the boundaries of the droplet, yet soft enough to allow the cells to move beyond the drop when exposed to the stimulus of a drop of attractant placed close by.

In order to have all the amoebae sensitive at the same time, the petri dishes containing the drops of amoebae (150 on each plate) were placed at 21 ± 1 C for about 3 hr and then at 5 C overnight.

When the plates were brought back to room temperature, 0.1-μliter droplets of the substance to be tested were placed near (less than 500 μm) the drop of amoebae, and the results were recorded after 45 and 120 min. If the cells moved out of the boundary of the drop, the fraction was scored positive. In cases

where the activity was especially weak, the fractions were reapplied five times at intervals of 5 min, thereby considerably increasing the sensitivity of the assay. The first recording of these results took place 5 min after the fifth application.

Purification. Purification involved column chromatography, paper chromatography, and paper electrophoresis. The bacterial water extracts were first chromatographed on Sephadex G-10 with a column having a 60-ml void volume. The active peak appeared between 66 and 78 ml after the void volume. The pooled active fractions of 20 such runs were rechromatographed four successive times on Sephadex G-10, and the resulting material was vacuum-evaporated to dryness at 50 C. It was then dissolved in distilled water and applied to paper for descending paper chromatography in a solvent system of *n*-butyl alcohol–acetic acid–water (4:1:5). The chromatograms were cut into strips, the strips were eluted, and the eluate was tested for activity. The active eluates were pooled, concentrated by vacuum evaporation (50 C), and applied to paper for electrophoresis. This was done in a pyridine-acetate buffer solution (10 ml of pyridine, 100 ml of acetic acid, 890 ml of water) at *p*H 3.9. Again, strips of the chromatograms were eluted, concentrated, and tested with the bioassay.

The active preparation was then put on a diethylaminoethyl (DEAE) Sephadex A-25 column in 0.1 M tris(hydroxymethyl)aminomethane buffer (*p*H 8.3) with a linear NaCl gradient from 0 to 1 M salt. The active fractions were again identified with the assay, collected, and adsorbed onto activated charcoal. The charcoal was separated from the supernatant fraction (which was inactive in the assay), and the active component was eluted from the charcoal with ethyl alcohol-NH_3-water (50:2:48). The preparation was brought to dryness by vacuum evaporation (50 C) and was chromatographed on ascending paper with three different solvent systems (Table 1).

Spectra. Ultraviolet spectra were obtained on a Cary 14 recording spectrophotometer.

RESULTS

It was possible to determine the approximate molecular weight by the identification of the active fractions from the Sephadex G-10 column. It was in the range of 200 to 400. From the paper electrophoresis at low *p*H, it was clear that the molecule had a negative charge. An ultraviolet absorption spectrum was obtained before passing the active fractions through DEAE Sephadex; the result was a spectrum with a pronounced λ maximum at 259 nm (see Fig. 1 in reference 7). When the *p*H was lowered from 7 to 2, the shape of the curve changed, but the λ maximum remained the same. The spectrum at *p*H 12 was identical with that at *p*H 7. The substance was heat-stable and retained its activity after boiling.

This information strongly suggested a derivative of adenine. Further purification on the DEAE Sephadex column and paper chromatography in the different solvent systems (Table 1) showed

TABLE 1. *R_F values of 3',5'-cyclic AMP, 5'-AMP, and E. coli extract*[a]

Solvent system	5'-AMP	3',5'-Cyclic AMP	*E. coli* extract
Isopropyl alcohol–ammonia–water (7:1:2)	0.05	0.35	0.35
n-Butyl alcohol–acetic acid–water (5:2:3)	0.17	0.30	0.30
Ammonium acetate–ethyl alcohol (3:7)	0.07	0.34	0.34

[a] R_F values for 5'-AMP and cyclic AMP were determined by scanning with ultraviolet light. The R_F of the active *E. coli* extract was measured by elution of 1-cm strips and testing with the bioassay.

conclusively that the material was 3',5'-cyclic AMP. Commercially obtained cyclic AMP gave identical R_F values in the various solvent systems.

A number of other naturally occurring nucleotides were tested for amoeba-attracting activity, but all examined were negative. This is true of 5'-AMP, 2'-(3')-AMP, adenosine diphosphate, adenosine triphosphate, cytidine monophosphate, cytidine triphosphate, uridine monophosphate, and inosine monophosphate.

Also, some analogues of cyclic AMP (kindly provided by A. R. Hanze of The Upjohn Co.) were tested. Tubercidin-5'-phosphate and its methyl ester were inactive, whereas tubercidin-3',5'-cyclic phosphate showed a clear ability to attract the amoebae, although it was less effective than cyclic AMP. Guanosine-3',5'-cyclic phosphate (kindly provided by G. Weimann of the Boehringer Co.), uridine-3',5'-cyclic phosphate, cytidine-3',5'-cyclic phosphate, thymidine-3',5'-cyclic phosphate, inosine-3',5'-cyclic phosphate, N^6-2'-*O*-dibutyryl-adenosine-3',5'-cyclic phosphate, and 5'-methylene adenosine cyclic phosphonate (kindly provided by J. G. Moffat of Syntex Laboratories) also were active, but less active than 3',5'-cyclic AMP.

DISCUSSION

It is known (3, 9) that *E. coli* and *Brevibacterium liquefaciens* secrete 3',5'-cyclic AMP into the surrounding medium. It is conceivable that this might be a common characteristic of bacteria, especially since various gram-positive and gram-negative bacteria attract amoebae (5). Some time ago we postulated that the amoebae oriented toward the bacteria as a food-seeking device and that the same mechanism might also be used, at a later stage, for the aggregation of the amoebae in the normal development of the cellular slime mold

(2). This hypothesis is supported by the fact that we now know that the slime mold also produces cyclic AMP (8).

The question of how the 3′,5′-cyclic AMP of the bacteria manages to orient the amoebae is a matter of considerable interest. The fact that some analogues of cyclic AMP are active might be a possible lead. Special attention should be drawn to the fact that all the analogues that show any ability to attract amoebae have the 3′,5′ ring; this seems to be an indispensable part of the molecule for orientation.

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