AN ADENOSINE-3′,5′-MONOPHOSPHATE ACTIVATED ADENYLATE CYCLASE IN THE SLIME MOLD *PHYSARUM POLYCEPHALUM*

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1. Introduction

The participation of cyclic AMP (cAMP)* in chemotaxis and differentiation has been established in the cellular slime mold *Dictyostelium discoideum*. In that organism, cAMP is the chemotactic agent that directs the movement of cells to aggregate into a multicellular organism [1]. The multinucleate acellular slime mold *Physarum polycephalum*, which grows as an expanding plasmodium and moves by a transfer of mass called 'shuttle streaming' [2], has been shown to be chemotactic toward certain metabolizable and non-metabolizable carbohydrates and amino acids [3]. Despite behavioral and morphological differences between cellular and acellular slime molds, the involvement of cAMP in sensory processes is very likely a unifying feature of the sensory systems of these organisms. *Physarum* provides an advantageous system for a study of molecular phenomena related to sensory transduction, not only because of convenience of culture and growth of synchronous cells having rapid and measurable motile responses but also because the behavioral and biochemical events that can be studied take place within a single giant cell, uncomplicated by population effects and intercellular interactions. Components of cAMP metabolism of *Physarum* have not been studied in any detail, although intracellular and extracellular cAMP phosphodiesterases [4] have been demonstrated in this organism, as have an unusual cAMP-inhibited protein kinase [5] and a polyamine-inhibited nuclear adenylate cyclase possibly involved in gene transcription [6]. In the present study, we report an adenylate cyclase from *Physarum polycephalum* that is activated by its product cAMP.

2. Materials and methods

Physarum polycephalum microplasmodia (originally obtained from spherules, strain M_3CV, generously supplied by Dr Harold Rusch of the University of Wisconsin) were maintained in shaken cultures in semi-defined citrate-buffered yeast–tryptone growth medium supplemented with hemin, as described by Daniel and Baldwin [7]. Cultures of microplasmodia, grown as dense suspensions of 30-ml volume in 500 ml trypsinizing Erlenmeyer flasks, were transferred by inoculation of 0.8–1.0 ml at 2-day intervals. Extracts were prepared from microplasmodia from mid-logarithmic growth phase (1.5–2.4 mg plasmodial protein/ml of culture medium). Microplasmodia were harvested by centrifuging for 1 min at approximately 300 × *g*. Cells were washed twice in 20 mM MES (2-(*N*-morpholino)ethane sulfonate) buffer, pH 5.8, and incubated without nutrients in this buffer for 3 h in a reciprocating shaker. The flasks were protected from light by wrapping them in aluminium foil. Cells were washed twice in MES buffer and the pellets resulting from the final centrifugation were frozen in liquid nitrogen. Before use, the lysates were thawed in one volume of 20 mM Tris–HCl buffer, pH 8.0,

* *Abbreviations:* cAMP, adenosine-3′,5′-monophosphate; cGMP, guanosine-3′,5′-monophosphate; Gpp(NH)p, 5′-guanylyl-imidodiphosphate; IBMX, 3-isobutyl-1-methylxanthine

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containing 1.5 M sucrose (Tris–sucrose solution) and further homogenized in a tight-fitting glass homogenizer with a ground-glass pestle for five strokes at 0°C. This latter homogenization was not required for activity but made the particles more uniform in size. Particulate and supernatant fractions were prepared from these homogenates by centrifugation at 2000 × *g* for 10 min. The pellet was resuspended in 'Tris–sucrose' and will be referred to as the particulate fraction. The 2000 × *g* supernatant fluid was centrifuged at 100 000 × *g* for 2 h. Both the particulate fraction and the 100 000 × *g* supernatant were stored in liquid nitrogen.

The standard assay mixture for adenylate cyclase in a final volume of 0.1 ml contained: 40 mM glycine buffer (pH 10), 1 mM [α^{32}P]ATP (20 mCi/mmol, Amersham or New England Nuclear), 10 mM $MgCl_2$, 0.2% bovine serum albumin (Sigma fraction V), 10 units of creatine phosphokinase, and 20 mM creatine phosphate. The reaction was started by the addition of 20–100 μg of cell fraction. Duplicate samples were incubated in a shaking water bath at 37°C for 5 min, unless otherwise noted, and the amount of cAMP formed from [α^{32}P]ATP was determined by the method of Salomon et al. [8] with the exception that the reactions were terminated with 0.1 ml of a reagent consisting of 2% sodium dodecyl sulfate, 10 mM ATP, 1 mM cAMP, and 10 mM EDTA, at pH 7.4. Assay blanks were prepared by adding enzyme after addition of the stopping reagent to the reaction. One unit of enzyme activity is defined as one pmol of cAMP formed per mg protein per 5-min incubation. Under the conditions described above the reaction was linear with time for at least 20 min and with protein concentration up to 1 mg/ml. The product of the reaction was shown to be cAMP by thin-layer chromatography on PEI plates using 0.3 M LiCl as solvent and by radioimmunoassay using the method of Harper and Brooker [9].

Phosphodiesterase activity, when measured in the particulate fraction, was assayed in reaction mixtures (final volume 0.5 ml) containing 25 mM Tris-HCl (pH 7.5), 25 mM imidazole (pH 7.5), 6 mM magnesium acetate, and 0.5 mM [^{14}C]cAMP (approximately 10 000 cpm, ICN Pharmaceuticals). Reactions were started by the addition of 50–100 μg of cell fraction. Duplicate samples were incubated at 37°C for 10 min. Reactions were stopped with EDTA and the cAMP hydrolyzed by the phosphodiesterase was assayed using snake venom and Dowex AG1-X8 (BioRad) columns as described previously [10]. Assay blanks were prepared by adding enzyme preparations after EDTA and snake venom. In a typical experiment 5–30% of the total substrate present was hydrolyzed. One unit of phosphodiesterase is defined as 1 nmol of cAMP hydrolyzed per mg protein per 10-min incubation.

Proteins were measured by the method of Lowry et al. [11], and radioactivity was measured on a Packard Tricarb scintillation counter using Instagel (Packard) as the scintillation cocktail.

Sources of materials were those mentioned elsewhere in the text and as follows: ATP, cAMP, cGMP, GTP, adenosine, creatine phosphate, creatine phosphokinase, dithiothreitol, 3-*O*-methyl-D-glucose, MES, and Tris were from Sigma; 5′-guanylyl-imidodiphosphate (Gpp(NH)p) was from ICN Pharmaceuticals; D-glucose was from Baker; sodium dodecylsulfate was from Fisher; 3-isobutyl-1-methylxanthine and morin were from Aldrich; and 8-bromo cAMP and 8-methylthio cAMP were a gift of Syntex Research (Palo Alto, CA).

3. Results

3.1. *Effect of starvation on adenylate cyclase activity*

Adenylate cyclase activity in *Physarum polycephalum* was present in both particulate and 100 000 × *g* supernatant fractions in a ratio of approximately 3:1. Adenylate cyclase activity was found to be influenced by the nutritional status of the organisms. Activity in 2000 × *g* particles was highest when microplasmodia from mid-logarithmic growth phase had been starved for 3–6 h, showing an increase of 5- to 10-fold over activity in unstarved cells and a subsequent decrease after 6 h of starvation (table 1). Starvation of the cells resulted in a concomitant increase in phosphodiesterase activity up to a period of 3 h. This was followed by a rapid decrease of enzyme activity.

3.2. *General properties of adenylate cyclase*

Characteristics of particulate adenylate cyclase (table 2) include a lack of stimulation by sodium fluoride or by either GTP or 5′-guanylyl-imidodi-

Table 1
Effect of starvation on adenylate cyclase and phosphodiesterase activities

Starvation (h)	Adenylate cyclase activity (pmol/mg protein/5 min)	Starvation (h)	Phosphodiesterase activity (nmol/mg protein/10 min)
0	2	0.5	46
1	10	1	76
3	16	2	98
6	21	3	147
9	8	4	82
20	8	6	84
		8	78

Representative experiments using 2000 × *g* particles are shown. Starvation of cells and assays of adenylate cyclase and phosphodiesterase activities were performed as described in the text

Table 2
Effects of various agents on adenylate cyclase activity

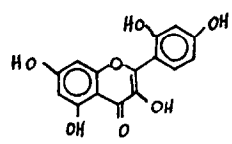
Addition to assay mixture	% Activity of complete system
None	100[a]
100 000 × *g* supernatant (particles omitted)	31
ATP omitted	0
$MgCl_2$ omitted	5
Creatine phosphokinase and creatine phosphate omitted	25
25 mM NaF	100
100 μM GTP	100
100 μM Gpp(NH)p	100
1 mM Adenosine	100
5 mM 5′-AMP	66
0.1 mM $CaCl_2$	100
0.1 mM 3-*O*-methylglucose	100
1 mM IBMX[b]	130
0.5 mM Morin[c]	20
10 μM cAMP	200
cGMP (10 μM, 50 μM, or 500 μM)	100
10 μM cAMP +10 μM cGMP	135
10 μM 8-bromo cAMP or 8-methylthio cAMP	100

Assays were performed as described in the text using 2000 × *g* particles, except for the indicated additions, deletions, or substitutions

[a] Average value with complete assay mixture for these experiments was 52 pmol/mg/5 min

[b] 3-Isobutyl-1-methylxanthine

[c] A flavonoid compound having the structure:



phosphate (Gpp(NH)p). The enzyme was not affected by the addition of 1 mM adenosine, 10 μM $CaCl_2$, or by 100 μM 3-*O*-methylglucose, which serves as a potent chemoattractant for this organism [3]. Slight inhibition by 5'-AMP was observed. Little or no activity was observed in the absence of ATP, Mg^{2+}, or an ATP-regenerating system. The pH optimum of the adenylate cyclase was 9.5–10.0 and the optimal temperature of incubation was 37°C. Enzyme activity was retained, and even slightly enhanced, by pre-incubation at 37°C for up to 30 min. Addition to the reaction mixture of the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine (IBMX) at 1 mM increased the cAMP formed by 30% in the particles but had no effect on the enzyme in the supernatant fluid.

While the presence of 1.5 M sucrose stabilized adenylate cyclase activity, sulfhydryl reagents and EDTA were not important for stability and also did not increase activity significantly when added to the reaction mixture. 0.2% bovine serum albumin was required in the reaction mixture to maintain stability (presumably because of high protease activities in the homogenates). Homogenization of the once-frozen and thawed extracts using a homogenizer with a ground-glass pestle did not decrease nor further increase activity from that obtained by freezing and thawing alone. More than one freeze-thaw cycle resulted in a loss of activity of at least 75%.

3.3. *Activation of adenylate cyclase by cAMP*

Addition of cAMP to the reaction mixture resulted in an increase in the adenylate cyclase activity. Concentrations as low as 1 μM cAMP caused enzyme activation. The K_a for cAMP is approximately 2 μM (fig.1). Activation by cAMP appears to increase the affinity of the enzyme for its substrate ATP. Figure 2 shows that the apparent K_m of the adenylate cyclase for ATP is 1 mM in the absence of cAMP, 0.75 mM in the presence of 2 μM cAMP, and 0.5 mM in the presence of 500 μM cAMP. Since enzyme activation by cAMP was measured by an increase in cAMP production, the question arose whether such an increase may be due to the added cyclic nucleotide providing a pool for the phosphodiesterase. This is unlikely, since the amount of cAMP hydrolyzed by particulate and extracellular phosphodiesterase in the presence of micromolar quantities of cAMP is not

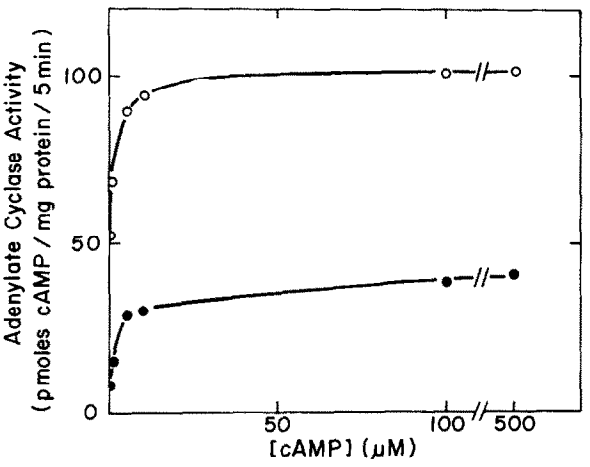


Fig.1. Activation of adenylate cyclase by cAMP. Assays were carried out as described in the text. The effect of cAMP was studied on adenylate cyclase in the particulate fraction (○) and in the supernatant fraction (●).

significantly different from that in the absence of added unlabeled cAMP (Kincaid and Mansour, unpublished results). cGMP (at 10, 50, or 500 μM) had no activating effect on the adenylate cyclase activity; however, it reduced activation of the enzyme by cAMP. cAMP analogues, 8-methylthio cAMP and 8-bromo cAMP, which are less readily hydrolyzed by phosphodiesterase in most systems, did not activate

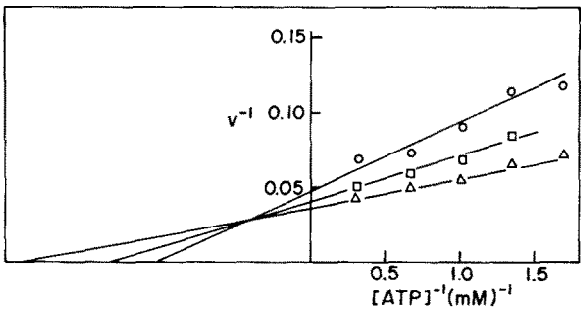


Fig.2. Lineweaver-Burk plot of adenylate cyclase activity with respect to substrate concentration. Assays were carried out as described in the text in the presence of the indicated concentrations of ATP and cAMP. Velocity (v) is expressed in units of adenylate cyclase activity (pmol cAMP per mg protein per 5-min incubation). Kinetics in the presence of increasing cAMP concentrations are shown: (○) no cAMP, (□) 2 μM cAMP, (△) 500 μM cAMP.

adenylate cyclase at a concentration of 10 μM, while 8-bromo cAMP caused 50% activation at a concentration of 100 μM.

3.4. *Inhibition of adenylate cyclase by a flavonoid*

The naturally occurring flavonoid morin was found to be a potent inhibitor of phosphodiesterase activity in *Physarum* (Kincaid and Mansour, unpublished data). This class of compound is of particular interest since, unlike other types of phosphodiesterase inhibitors, morin is also a potent inhibitor of adenylate cyclase activity in *Physarum* (table 2). Inhibition of adenylate cyclase by morin was found to be competitive with respect to ATP (fig.3) but noncompetitive with respect to cAMP (data not shown). While cAMP is the only activator for *Physarum* adenylate cyclase as yet known, 5′-AMP and morin are the only inhibitors of the enzyme that have been found. Neither compound is competitive with cAMP activation, and it is not clear whether the observed inhibition by 5′-AMP results from a direct effect on the enzyme. Kinetic studies of a competitive inhibitor of cAMP activation may aid in further elucidation of the mechanism of activation of *Physarum* adenylate cyclase by cAMP.

4. Discussion

The results reported in this paper demonstrate that the slime mold *Physarum polycephalum* possesses a particulate adenylate cyclase that is activated by cAMP. Previous work on intact *Dictyostelium discoideum* has suggested that the adenylate cyclase is activated by a cAMP/cGMP cascade [12] which acts as an amplifier in the generation of cAMP oscillations. No direct demonstration of adenylate cyclase activation by either cAMP or cGMP in *Dictyostelium* has as yet been reported. The present investigation identifies adenylate cyclase in *Physarum* as an enzyme that is activated by the product of its reaction, cAMP. The nature of activation by cAMP appears to be kinetic and not mediated through a protein-kinase-activated cascade. This is implied from the kinetic data which showed that cAMP lowers the K_m for ATP of adenylate cyclase in addition to increasing the V_{max}. Both properties are characteristic for a non-essential activator. Furthermore, previously reported work on protein kinase showed that the kinase in *Physarum* is inhibited by cAMP [5].

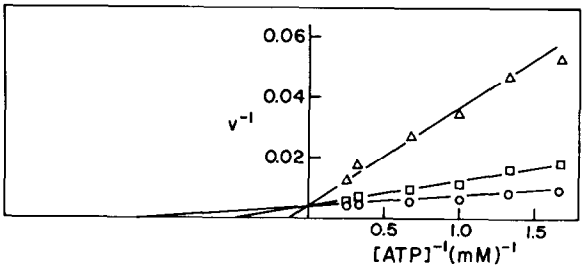


Fig.3. Lineweaver-Burk plot showing competitive inhibition of substrate kinetics by morin. Assays were carried out as described in the text in the presence of 5 μM cAMP and the indicated concentrations of ATP and morin. Velocity (v) is expressed in units of adenylate cyclase activity (pmol cAMP per mg protein per 5-min incubation). (○) no morin, (□) 100 μM morin, (△) 600 μM morin.

The regulation of adenylate cyclase from *Physarum polycephalum* by the reaction product cAMP has some interesting implications regarding the maintenance of cyclic nucleotide levels in this slime mold. Feedback activation of adenylate cyclase by increasing levels of its product would be consistent with an oscillatory accumulation of cAMP, such as that observed in *Dictyostelium discoideum*. This has already been observed also with *Physarum* (manuscript in preparation). Although the relationship between chemotaxis and the unusual control of adenylate cyclase by cAMP in *Physarum* is still speculative, it is interesting to note that conditions that were optimal for adenylate cyclase activation such as starvation, stage of growth, and specific culturing conditions are also optimal for demonstrating chemotaxis [3]. Furthermore, cAMP was shown in unpublished work of Kincaid and Mansour to act as a positive chemoattractant. The cAMP-activated adenylate cyclase in the cellular membrane may be an integral part of a communication system in the slime mold.

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References

[1] Konijn, T. M., Van De Meene, J. G. C., Bonner, J. T. and Barkley, D. S. (1967) Proc. Natl. Acad. Sci. USA 58, 1152–1154.
[2] Seifriz, W. (1937) Science 86, 397–398.
[3] Kincaid, R. and Mansour, T. E. (1978) Exp. Cell Res. in press.
[4] Murray, A. W., Spiszman, M. and Atkinson, D. E. (1971) Science 171, 496–498.
[5] Kuehn, G. D. (1971) J. Biol. Chem. 246, 6366–6369.
[6] Atmar, V. J., Westland, J. A., Garcia, G. and Kuehn, G. D. (1976) Biochem. Biophys. Res. Commun. 68, 561–568.
[7] Daniel, J. W. and Baldwin, H. H. (1964) in: Methods in Cell Physiology (Prescott, D. M. ed) vol. 1, pp. 9–41. Academic Press, New York.
[8] Salomon, Y., Londos, C. and Rodbell, M. (1974) Anal. Biochem. 58, 541–548.
[9] Harper, J. F. and Brooker, G. (1975) J. Cycl. Nucl. Res. 1, 207–218.
[10] Mansour, T. E. and Mansour, J. M. (1977) Biochem. Pharmacol. 26, 2325–2330.
[11] Lowry, O. H., Rosebrough, N. Y., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[12] Wurster, B., Schubiger, K., Wick, U. and Gerisch, G. (1977) FEBS Lett. 76, 141–144.