ADENYLATE CYCLASE ACTIVITY IN *DICTYOSTELIUM DISCOIDEUM* AMOEBAE AND ITS CHANGES DURING DIFFERENTIATION

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1. Introduction

Extracellular cAMP is a chemotactic agent of starved *Dictyostelium discoideum* cells [1,2]. The rhythmic excretion of cAMP by amoebae comprises the chemotactic signal which orients cell migration during the aggregation phase of their developmental cycle [3,4] and induces the differentiation of cells into aggregation-competent amoebae [5].

Current data suggest that the response of cells to the chemotactic signal is mediated by cell surface cAMP receptors, as evidenced by surface cAMP-binding sites [6]. Activation of these receptors results in an amplification of the signal: Intracellular cAMP levels increase approximately 20-fold within minutes after a pulse [7], most of which is then excreted [8,9]. Indirect evidence indicates that the increase in cellular cAMP levels influences the differentiation of cells to aggregation competence [10] while, obviously, the amounts of cAMP excreted by the cells determine the quality of the chemotactic signal. Therefore, the factor which couples the chemotactic and differentiation-inducing effects of extracellular cAMP is the enzyme adenylate cyclase. This communication describes an in vitro assay for the adenylate cyclase of *D. discoideum* amoebae and shows that a 40-fold increase in activity occurs during the differentiation of cells to aggregation competence.

2. Experimental

Unless indicated otherwise, enzyme activity was assayed in lysates from aggregation-competent cells. Ax-2 amoebae [11] were starved for 6 h in 17 mM phosphate buffer, pH 6.2, as described by Beug et al. [12], washed twice with 10 mM MES (2-(*N*-morpholino) ethane sulfonate) buffer, pH 6.2, and frozen as pellets in dry-ice-ethanol. Cells were thawed in 10 mM Tris, pH 8, 1 mM EDTA, 1 mM $MgCl_2$ and 1.5 M sucrose. Between 50–200 μg of lysate protein was added to a buffer containing 40 mM Tris pH 8, 0.5 mM [α-^{32}P]ATP (New England Nuclear, 80 Ci/mol), 10 mM KCl, 1 mMcAMP, 10 mM $MgSO_4$, 50 mM dithiothreitol, 0.3% BSA, 2.8 M phosphoenol pyruvate (Calbiochem.) and 0.6 μg pyruvate kinase. Triplicate samples were incubated at 27°C for 5 min. Any changes in the constituents of the buffer or the conditions of incubation are indicated in the legends to the figures and tables. [^{32}P]cAMP formed from [α-^{32}P]ATP was measured according to the procedure of Ramachandran [13] using neutral alumina columns.

3. Results and discussion

Experiments verifying that the reaction product measured in the in vitro assay for adenylate cyclase was cAMP are shown in fig.1. When digested with cAMP phosphodiesterase, or chromatographed on Domex 50 X 8 (H^+) or on polyetheyleneimine cellulose, the ^{32}P radioactivity eluted from the alumina columns behaved identically to added cAMP.

Summarized in table 1 are the effects of various compounds on adenylate cyclase activity measured in cell lysates. Whole cells could not catalyze the formation of cAMP suggesting that adenylate cyclase is not located on the outer surface of the cell membrane. No significant activity was observed in the absence of ATP, an ATP-regenerating system, or Mg^{2+}. At 10 mM

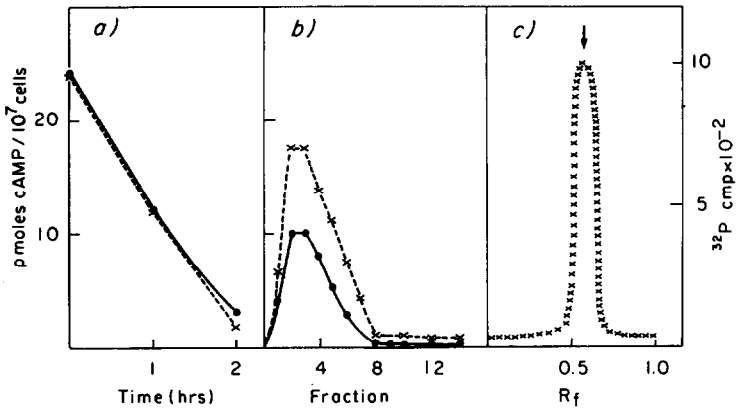

Fig.1. Identification of cAMP from alumina columns. (a) An aliquot of the eluate was counted for ^{32}P radioactivity. A corresponding number of pmoles of [^{3}H]cAMP was added to the remainder and the mixture incubated at 30°C with cAMP phosphodiesterase. At the indicated times the remaining number of pmoles of cAMP was determined. X–X ^{32}P, •–• ^3H. (b) An eluate with added [^{3}H]cAMP was chromatographed on Dowex 50X8 (H^+) as described by Krishna et al. [14]. X–X ^{32}P, •–• ^3H. (c) Samples were chromatographed on polyethyleneimine cellulose plates in isobutyric acid/ammonia/water (66:1:33 v/v) [13] with non-radioactive cAMP as a marker. Plates were scanned for ^{32}P radioactivity using the Chromelec 101 (Numelec, Versailles). Cyclic AMP was located by UV absorption. Arrow indicates the position of marker cAMP.

$MgSO_4$, the apparent K_m of the enzyme for ATP was between 0.2–0.5 mM (data not shown). App(NH)p, adenylimidodiphosphate, could substitute for ATP but only half the activity was observed at the ATP concentration used in the reaction mixture (0.5 mM). Dithiothreitol, a competitive inhibitor of *D. discoideum* phosphodiesterase [15], was added to the reaction mixture, as was cAMP, to minimize the hydrolysis of the cAMP synthesized during the incubation. In the absence of either compound, significant phosphodiesterase activity could be detected in the lysates, which probably accounts for the decrease in cAMP synthesis measured under these conditions. This is consistant with the observation that cyclic GMP, which inhibited phosphodiesterase almost as effectively as cAMP, could partially substitute for cAMP in the reaction mixture. Adenylate cyclase was not stimulated by by NaF nor by μmolar concentrations of either GTP or Gpp(NH)p. In the presence of 2 mM 5′-AMP, little enzyme activity could be detected, while 40% of the original activity remained when the same concentration of 5′-GMP was present in the reaction mixture. Since

Table 1
Effect of in vitro added compounds on adenylate cyclase activity

Reaction mixture	% Activity	Reaction mixture	% Activity
–Complete	100	+$CaCl_2$ (0.1 mM)	50
–cAMP	50	(1.0 mM)	–*
–DTT	68	+5′-AMP (0.02 mM)	78
–cAMP+cGMP (1 mM)	70	(0.2 mM)	50
–Phosphoenolpyruvate		(2.0 mM)	10
pyruvate kinase	20	+5′-GMP (0.2 mM)	85
–Mg^{2+}	10	(2.0 mM)	40
–Lysate+whole cells	–*	–ATP	–*
+EDTA (1 mM)	100	–ATP+App(NH)p	50
+EGTA (1 mM)	100		

*Below the limit of detection (1 pmol)

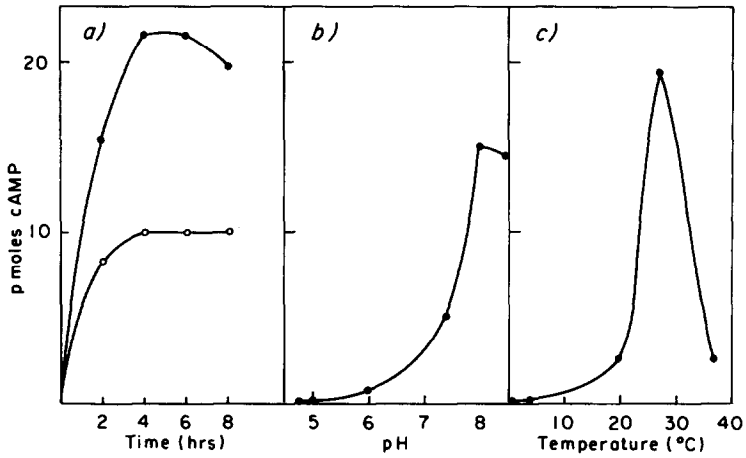

Fig.2. (a) Kinetics of cAMP synthesis at two protein concentrations: o–o 50 μg or •–• 100 μg cell lysate were added to incubation mixture and assayed as described. (b) Incubation mixtures were adjusted to the indicated pH and the formation of cAMP determined during 4 min incubations. (c) Mixtures were incubated at the indicated temperatures for either 2, 4 or 6 min. The relative amounts of cAMP formed were the same in all cases. (Data shown for 6 min incubation.)

cAMP induces phosphodiesterase in vivo [16,17] and 5′-AMP is the product of the phosphodiesterase, the effect of 5′-AMP on adenylate cyclase activity may be indicative of the mechanism by which enzyme activity is regulated in the cell. However until the enzyme is purified, such interpretations remain highly speculative. No effect of 1 mM EDTA or EGTA on adenylate cyclase activity was observed but the addition of 1 mM Ca^{2+} totally inhibited enzyme activity. The inhibition of enzyme activity by Ca^{2+} has been observed in various systems [18].

Figure 2a shows that adenylate cyclase activity was linear with time up to 4 min. The decrease noted after 6 min with the higher protein concentration indicates that phosphodiesterase, under these conditions, was still active. Maximal adenylate cyclase activity was observed at pH 8 (fig.2b) and at 27°C (fig.2c), the temperature above which cells do not grow. No activity was detected when incubations were performed at 37°C. Lysates preincubated for 6 min at either 37°C or 27°C and then assayed for adenylate cyclase activity showed respectively 0% and 50% the activity of the lysates which had been kept at 4°C. When lysates which had been pre-incubated at 37°C were mixed with those which had been kept at 4°C, and then assayed for cyclase activity, the activity measured could be accounted for by the activity found in the lysate kept at 4°C. It would appear that the loss of adenylate cyclase activity at 37°C (and the limited time of activity at 27°C) is not due to the production of a diffusible inhibitor, or loss of diffusible activator, but rather reflects the instability of the enzyme. Cell extracts prepared by either homogenization or sonication, and in the presence of protease inhibitors, showed the same kinetics and levels of cAMP synthesis as lysates prepared by freeze-thawing cell pellets as described in Experimental.

Table 2 indicates that changes in enzymatic activity accompany the differentiation of amoebae to aggregation competence. Cells harvested from exponentially growing cultures showed no significant adenylate cyclase activity. Activity was detectable after 2–3 h

Table 2
Changes in adenylate cyclase activity during cell differentiation

Culture condition	Adenylate cyclase activity
Exponential growth	(pmoles cAMP/10^7 cells)
	–*
Starved 1 h	–*
3 h	20
4 h	45
5 h	45

*Below the limit of detection (1 pmol)

of starvation and was maximum after 4–5 h, when cells expressed aggregation competence.

The adenylate cyclase described in the communication is strikingly different from that reported by Rossamondo and Sussman [19] who followed the conversion of [^{14}C]ATP into [^{14}C]cAMP by chromatography on Eastman chromotogram sheets. The activity they report was maximal at 37°C, linear with time until 10 min, retained inextracts preincubated at 37°C for 20 min and did not vary with the developmental state of the amoebae. The activity reported here is maximal at 27°C, linear with time until 4 min, rapidly lost in lysates preincubated at either 27°C or 37°C, and is developmentally regulated. The reasons for these differences are unclear but may be due to differences in assay conditions.

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References

[1] Bonner, J. T. (1967) The Cellular Slime Molds, 2nd Edn., Princeton University Press, Princeton, New York.
[2] Bonner, J. T., Barkley, D. S., Hall, EM., Konijn, T. M., Mason, J. W., O'Keefe, G., III and Wolfe, P. B. (1969) Dev. Biol. 20, 72.
[3] Shaffer, B. M. (1962) Advan. Morphogenesis 2, 109.
[4] Gerisch, G. (1968) in: Current Topics in Developmental Biology, (Monroy, A. and Moscona, A. eds) Vol. 3, p. 157, Academic Press, New York.
[5] Darmon, M., Brachet, P. and Pereira da Silva, L. H. (1975) Proc. Natl. Acad. Sci. USA 72, 3163.
[6] Malchow, D. and Gerisch, G. (1974) Proc. Natl. Acad. Sci. USA 71, 2423.
[7] Ross, W., Nanjundiah, V., Malchow, D. and Gerisch, G. (1975) FEBS Lett. 53, 139.
[8] Schaffer, B. M. (1975) 255, 549.
[9] Gerisch, G., Fronm, H., Huesgen, A. and Wick, U. (1975) Nature 255, 547.
[10] Klein, C. and Brachet, P. (1975) Nature 254, 432.
[11] Watts, D. J. and Ashworth, J. M. (1970) Biochem. J. 119, 171.
[12] Beug, J., Katz, F. E. and Gerisch, G. J. (1973) Cell. Biol. 56, 647.
[13] Ramachandran, J. (1971) Anal. Biochem. 28, 227.
[14] Krishna, G., Weiss, B. and Brodie, B. B., J. Pharmacol. Exp. Thes. 163, 379.
[15] Green, A. and Newell, P. (1975) Cell 6, 129.
[16] Klein, C. (1975) J. Biol. Chem. 250, 7134.
[17] Klein, C. and Darmon, M. (1975) Biochem. Biophys. Res. Commun. 67, 440.
[18] For review see (1972) Advances in Cyclic Nucleotide Research (Greengard and Robison, eds) North-Holland, Amsterdam.