ADENYLATE CYCLASES IN PHYSARUM POLYCEPHALUM: INHIBITION OF A NUCLEAR ENZYME BY POLYAMINES

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SUMMARY: Two distinct adenylate cyclase enzymes have been found in Physarum polycephalum. One is derived from isolated nuclei and is potently inhibited by an equimolar combination of the three polyamines, putrescine, spermidine, and spermine. The second enzyme is particulate, derived from the cytoplasmic compartment, and is insensitive to inhibition by the polyamines. These observations support a potential role for the polyamines in the control of adenosine 3',5'-monophosphate synthesis in P. polycephalum nuclei.

Cyclic adenosine 3',5'-monophosphate (cAMP) has been shown to enter prominently in the process of aggregation and development in the cellular slime mold, Dictyostelium discoideum (1-4). In contrast, in the multinucleated acellular slime molds such as Physarum polycephalum, the metabolic and regulatory roles of this cyclic nucleotide are virtually unexplored. Of the two enzymes which must participate in maintaining the levels of cAMP in living cells, namely adenylate cyclase and cAMP phosphodiesterase (5,6), only the latter has been demonstrated in P. polycephalum (7). In addition, a cAMP-inhibited protein kinase has been reported in this organism which presumably is involved in mediating some cyclic nucleotide effects (8). This paper reports preliminary characterization of two adenylate cyclases from vegetative plasmodia of P. polycephalum.

MATERIALS AND METHODS

A culture of P. polycephalum strain CL was used in this study which is an apogamic derivative from a progeny clone of the strain C50 (9,10). Shake-cultures of microplasmodia were grown and maintained as described earlier (11). Nuclei were isolated from 48-hr growth-cultures of microplasmodia by the method of Mohberg and Rusch (12), and were subsequently washed twice with 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2 \cdot 6H_2O$, and 5 mM 2-mercaptoethanol (buffer

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A) to remove residual Triton X-100. After each wash, nuclei were collected by centrifugation at 1,400 x g. Crude nuclear extracts were prepared at 4° C by suspending ~1-2 gm of wet-packed isolated nuclei in 10 ml of buffer A and passing them twice through a French pressure cell at 18,000 psi. The extruded mixture was initially dialyzed 20 hr against 100 times its volume of buffer A, then centrifuged at 30,000 x g for 20 min to remove a small amount of insoluble debris. In some experiments, the final centrifugation was performed at 200,000 x g for 2 hr. After this treatment, all adenylate cyclase activity was found in the supernatant fraction.

A particulate adenylate cyclase from the cytoplasmic compartment was prepared at 4°C by removing whole nuclei from homogenized plasmodia (12) in 0.01 M Tris·HCl (pH 7.2), 0.25 M sucrose, and 0.01 M $CaCl_2$, yielding a supernatant fraction that was subsequently dialyzed against buffer A. The dialyzed fraction was centrifuged at 30,000 x g and the resulting supernatant fraction was recentrifuged at 200,000 x g for 2 hr. The soluble fraction from this final spin, contained 7% of the adenylate cyclase units assayed in the corresponding sedimented,particulate material. This sedimented fraction contained the particulate or cytoplasmic adenylase cyclase activity. None of the other sedimented or supernatant fractions, leading to the isolation of this particulate adenylate cyclase, contained significant cyclase activity.

Adenylate cyclase assays were conducted by the method of Saloman et al. (13), with the addition of 20 mM theophylline. Other modifications are described in Results. Reactions were terminated by addition of 15 µl of 0.2 N HCl and boiling for 10 min. cAMP produced was determined by the competitive binding assay of Gilman (14) using a cAMP binding-protein partially purified from bovine kidney (15). Protein was determined by the method of Lowry et al. (16). All enzyme units are expressed as picomoles of product produced per min of reaction time; specific activity is defined as units per mg of protein.

RESULTS

Table 1 shows the reaction requirements of the adenylate cyclase assayed in crude nuclear extracts. Concentrations for ATP and Mg^{2+} were established by prior determination of the Michaelis constants (K_m) from concentration-dependent rate studies. The K_m for ATP at 10 mM Mg^{2+} was 6.4×10^{-5} M; that for Mg^{2+} at 2 mM ATP was 5.2×10^{-4} M. Activity was also supported by Mn^{2+}, in place of Mg^{2+}, with a K_m of 4.9×10^{-4} M. Calcium ion, Ca^{2+}, was competitively inhibitory with respect to Mg^{2+} (data not shown). Neither an ATP-generating system nor NaF were found to appreciably affect the cyclase activity and were omitted from subsequent assays. Theophylline was essential to protect against hydrolysis of cAMP by a phosphodiesterase found to be present with a specific activity of approximately 55 picomoles/min/mg protein.

Table 2 shows the effect of three aliphatic polyamines, alone and in combination, on the nuclear adenylate cyclase activity. Single and dual

Table 1. Reaction Requirements for Adenylate Cyclase Assayed in Crude Nuclear Extracts from Physarum polycephalum.[a]

Component	Units (picomoles/min)
Complete[b]	6.04
Minus creatine phosphate and creatine phosphokinase	6.24
Minus ATP	0.24
Minus NaF	6.84
Minus theophylline	0.44

[a]Reactions contained 0.14 mg of protein.
[b]The complete reaction mixture contained 2 mM ATP, 10 mM $MgCl_2 \cdot 6H_2O$, 20 mM theophylline, 25 mM Tris (pH 7.5), 20 mM creatine phosphate, 10 units of creatine phosphokinase, and 20 mM NaF.

Table 2. Effect of Individual and Combined Mixtures of Polyamines on a Nuclear Adenylate Cyclase from Physarum polycephalum.[a]

Addition	Units (picomoles/min)	Percent Inhibition
None[b]	7.63	0
1 mM putrescine	6.36	16.6
1 mM spermidine	6.57	13.9
1 mM spermine	5.73	24.9
0.5 mM putrescine + 0.5 mM spermidine	4.07	46.6
0.5 mM putrescine + 0.5 mM spermine	3.26	57.3
0.5 mM spermidine + 0.5 mM spermine	5.00	34.5
0.3 mM putrescine + 0.3 mM spermidine + 0.3 mM spermine	0	100

[a]Reactions contained 0.10 mg of protein.
[b]The starting reaction components for the assay were described in Materials and Methods.

combinations of these compounds produced slight to moderate inhibition. However a combination of all three polyamines, each at 0.3 mM, completely inhibited the enzyme. The experiments shown in Table 2 tested each combination or single inhibitor at a total concentration near 1 mM polyamine. However,

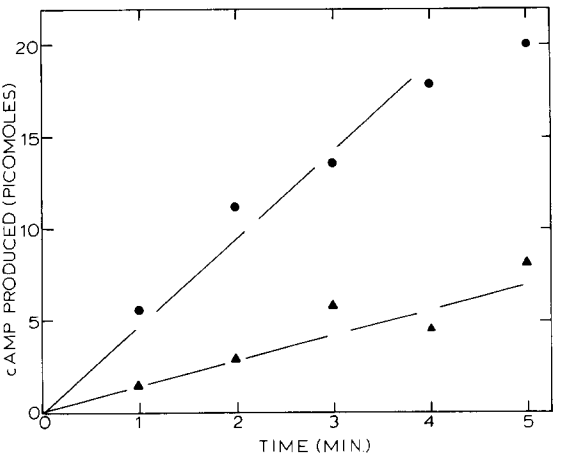

Fig. 1. The effect of polyamines on cAMP synthesis by a nuclear adenylate cyclase from Physarum polycephalum. Adenylate cyclase assays were conducted, as described in the text, in the absence (circles) and presence (triangles) of a combination of 0.3 mM putrescine, 0.3 mM spermidine, and 0.3 mM spermine. All reaction mixtures contained 0.11 mg of protein.

other experiments not shown here, revealed that lower polyamine concentrations alone and in dual combinations, were even less inhibitory.

Figure 1 shows that time-dependent synthesis of cAMP by the nuclear adenylate cyclase, in an enzyme preparation different from that used in Table 2, remained linear during the course of 5-min assays in both the presence and absence of a mixture of the three polyamines, each at 0.3 mM concentration. Figure 2 shows an experiment in which the activities of the cytoplasmic adenylate cyclase, and that associated with the nuclei were tested in the presence of increasing total concentrations of equimolar mixtures of the three polyamines. Again the soluble, nuclear enzyme was inhibited 94% by a combination of 0.33 mM of each polyamine (1 mM total polyamine concentration), whereas the particulate, cytoplasmic enzyme was insensitive up to 1 mM. In both cases where polyamine sensitivity and insensitivity were encountered, assays were conducted with radioactive polyamines. Subsequent isolation of the radioactive polyamines, by paper strip electrophoresis (17), from normal assays and control assays lacking the addition of enzyme, yielded comparable recoveries of the

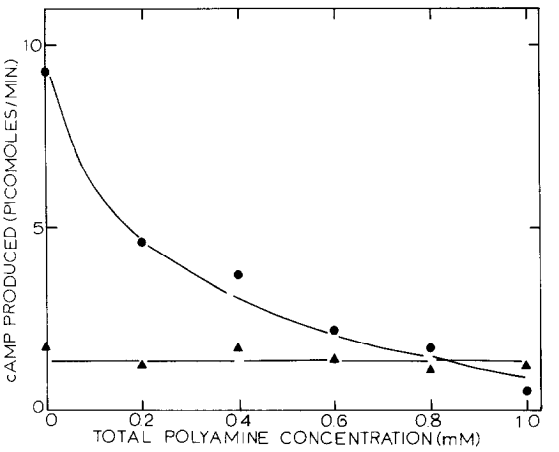

Fig. 2. The effect of polyamine concentrations on the activity of nuclear (circles) and cytoplasmic (triangles) adenylate cyclases from Physarum polycephalum. Equal concentrations of putrescine, spermidine and spermine were added to adenylate cyclase reaction mixtures to give total polyamine concentrations ranging from 0.2 to 1.0 mM. Assays measuring activity of the cytoplasmic adenylate cyclase contained 0.030 mg of protein; assays of nuclear adenylate cyclase contained 0.068 mg of protein.

starting ligands. This established that the polyamines were not metabolized during the adenylate cyclase assays and that oxidation products of the polyamines were not the mediators of the effect observed on adenylate cyclase activity (18). Polyamines at concentrations up to 1 mM did not affect the cAMP binding assay or the activity of the phosphodiesterase enzyme under the conditions of the adenylate cyclase assay described.

DISCUSSION

The most striking observations from these studies is the severe synergistic inhibition of a combination of three polyamines on a soluble, nuclear adenylate cyclase and that only this soluble enzyme is sensitive to these ligands. In contrast, the particulate, cytoplasmic adenylate cyclase is unaffected. The extent of inhibition observed by specific polyamine concentrations varied with different enzyme preparations (compare Fig. 1 and Table 2). However in no case was less than 70% inhibition observed at 1 mM total poly-

amine. Such varied sensitivity to modifiers could reflect cell cycle dependency or enzyme alterations associated with the rate or phase of growth (19,20, 21). To our knowledge, these studies represent the first demonstration that the polyamines can exert a direct effect on an adenylate cyclase without the mediation of an intact membrane system (22,23,24,25). These observations argue in favor of a potential role for the polyamines in the control of cAMP synthesis in P. polycephalum nuclei. As such, these compounds may enter prominently in controlling a host of nuclear phosphorylative events involved in gene transcription (26,27) and suggests a means by which they control their own synthesis (see discussion below).

The observations here allow suggestions of probable and testable biochemical roles by which the polyamines in other cell types may function as growth regulators (28,29). Polyamines are normal constituents of most cells ranging in concentrations between 0.25-2.5 mM for putrescine, and 1-2 mM for spermidine and spermine. Within P. polycephalum plasmodia, moreover, we have determined levels as high as 7 mM putrescine, 3 mM spermidine, and 7 mM spermine (30). These concentrations are well within the range for the effects we have observed on the nuclear adenylate cyclase. There is growing evidence from studies with several cell types that the induction of ornithine decarboxylase (ODC), the first unique enzyme in polyamine biosynthesis, requires cAMP (31, 32,33). Recent work has also shown that the polyamines control the induction of ODC by an unidentified process either at the transcriptional level or by destruction of the synthesized enzyme (34,35). As potential modifiers of adenylate cyclase, the polyamines could function as determinants of nuclear cAMP levels. In addition, they could control their own synthesis by modulating nuclear cAMP levels, and regulating the cAMP-dependent induction of ODC. Thus, a finely controlled regulatory loop would maintain both polyamine and nuclear cAMP levels in a poised, steady-state condition (36). The exceptionally rapid turnover (synthesis and degradation) rates for ODC reported in hepatectomized rate liver (11 min) (37) and P. polycephalum (14 min) (38,39)

are consistent with such a tightly controlled loop. Additional regulatory parameters assuredly would be superimposed on this system.

It may be signficant to note that usually factors that decrease cAMP levels stimulate the proliferation of cells growing in culture, while factors that increase cAMP levels cause a decrease in cellular growth rate (19). The correlation of high polyamine levels with neoplastic growth is well established (28). Over production of polyamines might be a condition of the proliferative state, either resulting in or caused by decreased cAMP levels.

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