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A 5′-Adenosine Monophosphate-Dependent Adenylate Cyclase and an Adenosine 3′:5′-Cyclic Monophosphate-Dependent Adenosine Triphosphate Pyrophosphohydrolase in *Dictyostelium discoideum*

(chemotaxis/enzyme regulation/phosphodiesterase/5′-nucleotidase/*Polysphondylium violaceum*)

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ABSTRACT **Cell aggregation in *Dictyostelium discoideum* appears to involve the production and detection of 3′:5′-cyclic AMP. Two pertinent catalytic activities have been studied in concentrates from *D. discoideum* purified 50- to 100-fold. They are (*i*) adenylate cyclase and (*ii*) ATP pyrophosphohydrolase. ATP pyrophosphohydrolase activity converts ATP to 5′-AMP and pyrophosphate. The presence of 5′-AMP is an absolute requirement for adenylate cyclase activity. The presence of 3′:5′-cyclic AMP is an absolute requirement for ATP pyrophosphohydrolase activity. Both activations, particularly that of ATP pyrophosphohydrolase, show narrow ranges of specificity and display significant cooperativities.**

After growth, the cells of *Dictyostelium discoideum* migrate in directed fashion toward central collecting points, enter onto organized multicellular aggregates, and construct fruiting bodies. This directed movement is the result of a chemotactic attraction exerted upon outlying individual cells by the nascent aggregative center and its radiating cell streams (1, 2), and appears to involve the production and detection of 3′:5′-cyclic adenosine monophosphate (cAMP) (3).

In order to understand the biochemical basis of this chemotactic response, attempts have been made to identify and characterize some of the macromolecular elements that might be involved. Thus, two 3′:5′-cAMP phosphodiesterase activities have been reported, the first with a molecular weight of about 132,000 and a K_m of 2 mM (4) and a second with a molecular weight of about 65,000 and a K_m of 15 μM (4–6).

In our previous communication we reported the presence of an adenylate cyclase activity in crude extracts of *D. discoideum* and a cAMP-dependent ATPase (7). We have since characterized the products of the latter reaction as adenosine 5′-monophosphate (5′-AMP) and pyrophosphate (PP_i). Hydrolases of this type have been reported in bull seminal fluid (8) and snake venom (9), and were termed "PP-forming ATPase" and "α,β-ATPase." The designation, ATP pyrophosphohydrolase, would seem to be more appropriate, and we use it hereafter.

The experiments we shall describe, using a partially purified (50- to 100-fold) preparation of the cyclase and pyrophosphatase, indicate that both enzymes have interesting regulatory properties that raise the possibility that they may function *in vivo* in a regulatory loop during the aggregation process.

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MATERIALS AND METHODS

Organisms. Two strains of *D. discoideum* were used. Ax-3 is derived from the parent stock, NC-4, and is adapted for growth in axenic medium (10). R-46 is a mutant with altered aggregative performance and very low phosphodiesterase activity (less than 1% of that of the wild type). The third organism is a related species, *Polysphondylium violaceum.*

Enzyme Purification. Cells were grown and harvested as described (7) and stored in frozen pellets. After they were thawed, the pellets were suspended in buffer VI [20 mM Tris·HCl–50 mM NaCl (pH 7.4)], treated in a Branson sonifier (1 min at 2 amp), and centrifuged at 30,000 × *g* for 30 min. The supernatant was centrifuged at 100,000 × *g* for 3 hr. Solid ammonium sulfate was added to the supernatant fraction to obtain the protein that precipitated between 40 and 65% saturation. This material was dissolved in and dialyzed against buffer VI for 4 hr, then applied to an agarose (6% Bio-gel A5) column (11) and eluted with the same buffer. The effluent was monitored with a flow cell and recorder (LKB) at 280 nm and collected. ^3H_2O was added to the applied sample for measurement of the included volume. All operations were done at 4°.

Enzyme Assays. The cyclase and pyrophosphohydrolase were measured by following the conversion of [^{3}H]ATP to either cAMP or 5′-AMP. After incubation at 37°, the components of the reaction mixture were separated by thin-layer chromatography as described (7). This procedure separates ATP, 5′-AMP, cAMP, and adenosine. The reaction mixture contained, in a final volume of 100 μl: 2 μmol of Tris·HCl (pH 7.4), 17 nmol of [^{3}H]ATP (2×10^4 cpm/nmol), 10 μmol of NaF, and various amounts of either 5′-AMP or cAMP. The cAMP phosphodiesterase was assayed by following the conversion of [^{3}H]3′:5′-cAMP to 5′-AMP. 5′-Nucleotidase activity was determined by measurement of the conversion of [^{3}H]5′-AMP to adenosine. Under these conditions, the initial reaction rates were directly proportional to enzyme concentration.

Demonstration of ATP Pyrophosphohydrolase. The use of a substrate mixture containing [^{3}H]ATP and [^{32}P]ATP labeled only in the gamma position demonstrated that 5′-AMP and PP_i are produced in equimolar quantities. Addition of excess unlabeled ADP was without effect. The concentrations of unlabeled cAMP necessary to activate the enzyme were also

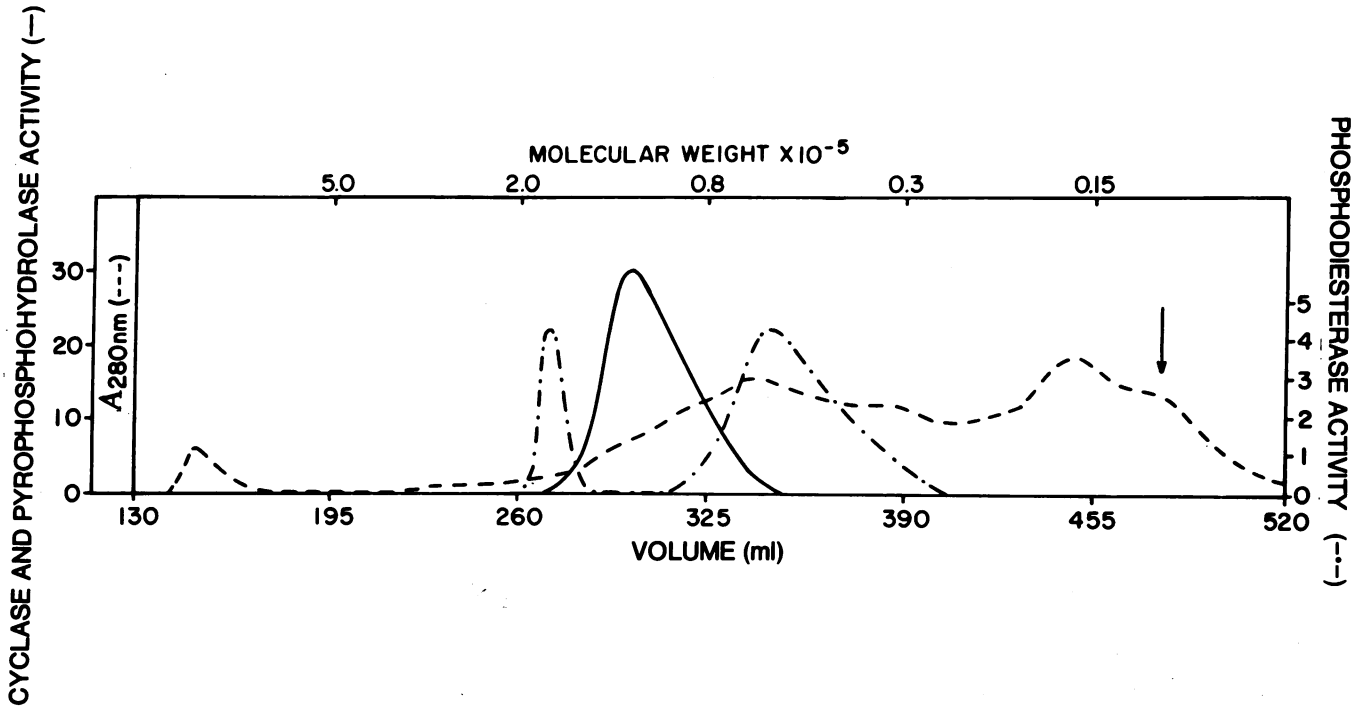

FIG. 1. Chromatography of a preparation of strain Ax-3 on a 2 × 170 cm column of 6% agarose. About 85 mg of protein was applied in 3 ml to the column. The column was eluted at 13 ml/hr, and absorbance was measured continuously in the effluent. Phosphodiesterase, ATP pyrophosphohydrolase, and adenylate cyclase activities were measured in the effluent fractions and recorded in arbitrary units. *Arrow* indicates included volume.

sufficient to rule out the possibility that the labeled 5′-AMP was coming from cAMP. Furthermore, no cAMP phosphodiesterase activity could be detected (in the purified fraction used) nor any nonspecific phosphatase activity (with *p*-nitrophenylphosphate as substrate).

Materials. [^{3}H]ATP (13.4 Ci/mmol), [^{3}H]3′:5′-cAMP (21 Ci/mmol), and [^{3}H]5′-AMP (12.6 Ci/mmol) were obtained from New England Nuclear Corp. All unlabeled nucleotides and nucleosides were from Sigma; cellulose chromatographic sheets were from Eastman Kodak. All other chemicals and solvents were reagent grade or better. Agarose was from Bio Rad Laboratories. Proteins were determined by the Lowry *et al.* procedure, with bovine-serum albumin as standard (13).

RESULTS

Removal of Phosphodiesterase Activity. Fig. 1 shows the profile of material absorbing at 280 nm and the distribution of enzyme activities in the agarose column effluent (see *Methods*). The adenylate cyclase and ATP pyrophosphohydrolase activities were eluted together in an effluent volume that would correspond to a globular protein of 100,000 ± 10,000 daltons. By this criterion the cyclase appears similar to the cyclase of *Escherichia coli* (14). The intracellular phosphodiesterase activity was eluted in two separate fractions. One appeared at an effluent volume that would correspond to a globular protein of 60,000 daltons, similar to the enzyme reported by Gerisch and coworkers (5) and Chassy (4).

The fractions containing the maximum cyclase and pyrophosphohydrolase activities were pooled and concentrated by positive pressure filtration (Amicon Ultrafiltration Membrane). These fractions showed about 50- to 100-fold increases in specific activity over that found in crude sonicates. Phosphodiesterase activity was not detectable in these concentrates. The 5′-nucleotidase activity, though present, was completely inhibited by the presence of fluoride (12). Consequently, the cyclase and pyrophosphohydrolase activities could be assayed unequivocally by measurement of the accumulations of the respective reaction products, cAMP and 5′-AMP.

5′-AMP Dependence of the Adenylate Cyclase. Preliminary purifications of the cyclase indicated a requirement for an activator of small molecular weight that could be met by addition of 5′-AMP to the reaction mixture. The kinetics of cAMP production in the presence of different concentrations of 5′-AMP are shown in Fig. 2A. The rates were constant for about 20 min and then diminished progressively, though never completely during the next 20 min. Several trivial explanations of this diminution have been eliminated. Thus, it is not due to disappearance of the activator, 5′-AMP, or the product, cAMP, since either compound, suitably labeled and added to the reaction mixture, could be quantitatively recovered after 40 min of incubation. The recovery of added 5′-AMP also ruled out the possibility that it contributes stoichiometrically to the reaction. (Moreover, separate experiments failed to reveal the presence of adenylate kinase activity in these fractions.) The enzyme itself is not unstable at 37°, since prior incubation even at 45° for 10 min did not reduce its activity detectably. Addition of fresh substrate or complete reaction mixture after 20 min did not accelerate the reaction.

In Fig. 3*A* the initial rates of cAMP production are plotted as a function of 5′-AMP concentration. The relationship shows a significant degree of cooperativity. Purified adenylate cyclase from mutant R-46 of *D. discoideum* and from another

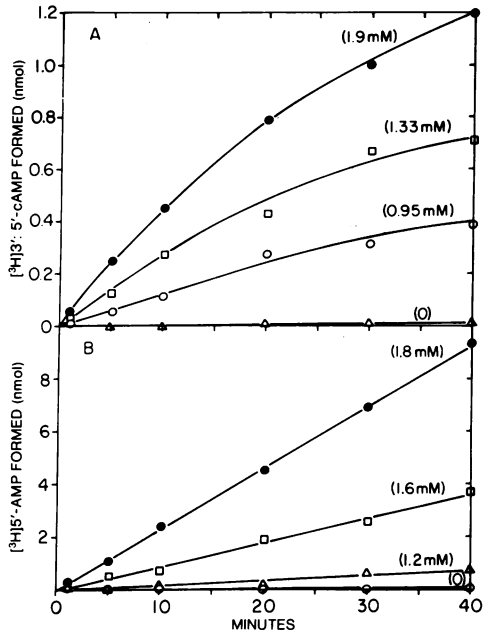

FIG. 2. (*A*) Time course of the reaction catalyzed by adenylate cyclase at different concentrations of 5'-AMP. The reaction mixture contained 35 μg of enzyme protein and other components, as described in *Methods*. The procedure for determination of [^{3}H]cAMP has been described (7). (*B*) Time course of the reaction catalyzed by ATP pyrophosphohydrolase (2 μg) at different concentrations of cAMP.

genus, *Polysphondylium violaceum*, responded to 5'-AMP in precisely the same fashion.

cAMP Dependence of ATP Pyrophosphohydrolase. Fig. 2*B* shows the kinetics of 5'-AMP production by the pyrophosphohydrolase in the presence of different concentrations of cAMP. The rates are linear over at least 40 min. In Fig. 3*B*, the rates are plotted as a function of cAMP concentration. The activation displays a remarkable degree of cooperativity. It should be emphasized that the product was completely stable under the assay conditions used. Hence, the sharpness of the curve is not an artifact due to insensitivity of the assay. Control experiments with labeled cAMP showed that this compound does not contribute stoichiometrically to the reaction. Fig. 3*B* also indicates that the ATP pyrophosphohydrolase purified from mutant R-46 and from *P. violaceum* showed the same pattern of cAMP dependence.

TABLE 1. *Effect of purine nucleotides on adenylate cyclase activity*

Nucleotide	Concentration (mM)	Concentration (nmol/30 min)
5'-AMP	1.9	1.10
	0.8	0.50
5'-GMP	1.7	0.34
	0.85	0.15
2'- and 3'-AMP	1.8	0
	0.9	0
ADP	1.5	0
	0.75	0
5'-dAMP	2.10	0.90
	1.05	0.50
5'-IMP	1.9	0
	0.8	0

Standard reaction mixtures containing the nucleotides at the specified (final) concentrations were incubated with 35 μg of enzyme protein present at 37° for 30 min. The mixture was then analyzed for 3':5'-cAMP content.

The specific activity of pyrophosphohydrolase in the purified concentrates was about 100-fold greater than that of the adenylate cyclase, the same difference as was encountered in crude extracts (7).

Specificities of the Activations. Both activations display narrow ranges of specificity. Table 1 shows that for the adenylate cyclase, 5'-AMP was as active as 5'-AMP at equimolar concentrations, while 5'-GMP was about one-third as active. 5'-IMP was completely inactive and, in fact, inhibited the 5'-AMP activation (Fig. 4) in a manner that suggests that the inhibition is competitive.

The specificity range for cAMP activation of the pyrophosphohydrolase is especially narrow. Neither 2':3'-cAMP

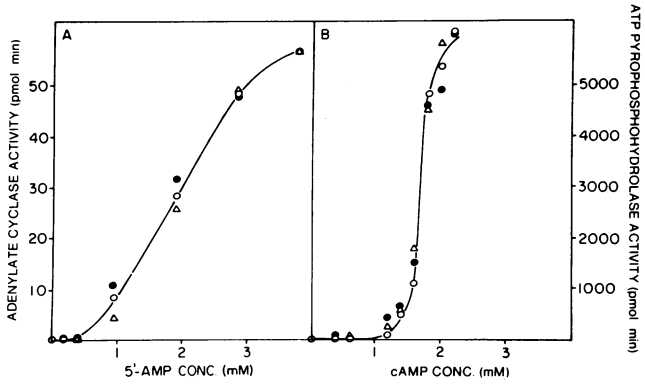

FIG. 3. The effect of 5'-AMP on the activity of adenylate cyclase (*A*) and the effect of cAMP on the activity of ATP pyrophosphohydrolase (*B*) for Ax-3 (○), R-46 (△), and *P. violaceum* (●). All data points were normalized to the data obtained with strain Ax-3. The standard reaction mixtures with various amounts of nucleotide was used with 35 μg of protein (*P. violaceum*).

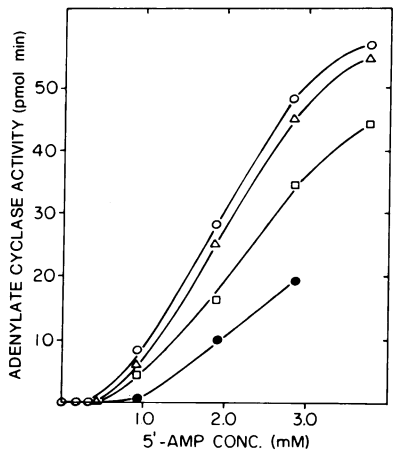

FIG. 4. The effect of addition of various amounts of 5'-IMP on the stimulation of adenylate cyclase by 5'-AMP. Reaction mixtures contained 35 μg of protein. No 5'-IMP (○); 0.95 mM (△); 1.90 mM (□); and 2.85 mM (●).

TABLE 2. *Effect of purine cyclic nucleotides on ATP pyrophosphohydrolase activity*

Cyclic nucleotide	Concentration (mM)	Activity (nmol/30 min)
3′:5′-cAMP	2.0	148.25
	1.0	9.54
2′:3′-cAMP	2.0	0
	1.0	0
3′:5′-cGMP	2.0	0
	1.0	0

Standard reaction mixtures containing the nucleotides at the specified (final) concentrations were incubated with 35 μg of enzyme protein at 37° for 30 min. The mixture was then analyzed for 5′-AMP content.

nor 3′:5′-cGMP could activate the enzyme detectably (Table 2).

DISCUSSION

The chemotactic complex that operates during cellular slime-mold aggregation presents at least two puzzling features:

(*i*) A relay system seems to be operative, i.e., cells, excited by the chemotactic agent, then produce it and thereby attract other cells (15).

(*ii*) In *D. discoideum*, concentric rings of responding cells move periodically toward the aggregative center in a concerted pattern that has a characteristic frequency and path length alterable by mutation (16).

If the adenylate cyclase and the ATP pyrophosphohydrolase do in fact constitute a regulatory loop that functions during aggregation, the first puzzling feature noted above can be at least partly accounted for and, by extension, so can the second. For example, if a cell were exposed to a pulse of exogenous cAMP, its pyrophosphohydrolase would be activated and would produce 5′-AMP. This 5′-AMP would in turn activate the cyclase to produce cAMP, of which some, at least, would be exported; thus a cell exposed to cAMP would make cAMP. The intracellular phosphodiesterase and 5′-nucleotidase might then function together to remove the cAMP and 5′-AMP and thereby influence the kinetics of cAMP synthesis in such a way as to insure its production and secretion in the form of pulses. The extreme cooperativity of the pyrophosphohydrolase activity may cause it to act as an "on–off" switch and might lead to the entrainment of all the cells in a restricted zone into a cyclic pattern of cAMP production, thereby leading to the observed pulsation of cell movements. This model also raises the possibility that exogenous cAMP and 5′-AMP (produced through the action of an extracellular phosphodiesterase) may conceivably act jointly to initiate and reinforce the chemotactic attraction. Early experiments did indeed indicate that the chemotactic activity of extracellular fluid from nascent aggregates could be approximated by mixtures of two components separated fron that fluid by paper chromatography. The chromatographic behavior of the two components observed in the earlier study indicates that they were almost certainly cAMP and 5′-AMP (17). It should also be noted that a mutually reinforcing regulatory loop of this kind might conceivably act in other organisms for other purposes.

Finally, the regulatory properties of these two enzymes raise several primarily biochemical questions. Are the two catalytic activities physically separable? What are the molecular bases of the activations, particularly with respect to the observed cooperativities?

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1. Runyon E. H. (1942) "Aggregation of *Dictyostelium* to form a multicellular body," *Collecting Net* **17**, 88–90.
2. Bonner, J. T. (1947) "Evidence for the formation of cell aggregated by chemotaxis," *J. Exp. Zool.* **106**, 1–26.
3. Konijn, T. M., Van de Meene, J. G. C., Bonner, M. T. & Barckley, D. S. (1967) "The acrasin activity of adenosine 3′:5′-cyclic phosphate," *Proc. Nat. Acad. Sci. USA* **58**, 1152–1154.
4. Chassy, B. M. 1972 "Cyclic nucleotide phosphodiesterase in *D. discoideum:* Interconversion of two enzyme forms," *Science* **175**, 1016–1018.
5. Gerisch, G., Malchow, D., Riedel, V., Muller, E. & Every, M. (1972) "Cyclic AMP phosphodiesterase and its inhibitor in slime mold development," *Nature* **235**, 90–92.
6. Pannbacker, R. & Bravard, L. (1972) "Phosphodiesterase in *D. discoideum*," *Science* **175**, 1014–1015.
7. Rossomando, E. F. & Sussman, M. (1972) "Adenyl cyclase in *D. discoideum*," *Biochem. Biophys. Res. Commun.* **47**, 604–609.
8. Heppel, L. A. & Hilmoe, R. J. (1953) "Mechanism of enzymatic hydrolysis of ATP," *J. Biol. Chem.* **202**, 217–226.
9. Johnson, M., Kaye, M. A. G., Hems, R. & Krebs, H. A. (1953) "Enzymatic hydrolysis of adenosine phosphates by cobra venom," *Biochem. J.* **54**, 625–629.
10. Cocucci, S. M. & Sussman, M. (1970) "RNA in cytoplasmic and nuclear fractions of cellular slime mold amoebae," *J. Cell Biol.* **45**, 399–407.
11. Rossomando, E. F. & Piez, K. A. (1970) "Molecular sieve chromatography of myosin on agarose columns," *Biochem. Biophys. Res. Commun.* **40**, 800–807.
12. Heppel, L. A. & Hilmoe, R. J. (1951) "Purification and properties of 5′-nucleotidase," *J. Biol. Chem.* **188**, 665.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) "Protein measurement with the Folin phenol reagent," *J. Biol. Chem.* **193**, 265–275.
14. Tao, M. & Huberman, A. (1970) "Some properties of *E. coli* adenyl cyclase," *Arch. Biochem. Biophys.* **141**, 236–240.
15. Schaffer, B. M. (1957) "Aspects of aggregation in cellular slime molds," *Amer. Natur.* **91**, 19–35.
16. Gerisch, G. (1968) "Cell aggregation and differentiation in *Dictyostelium*," *Curr. Top. Develop. Biol.* **3**, 157–197.
17. Sussman, M., Lee, F. & Kerr, N. S. (1956) "Fractionation of acrasin," *Science* **123**, 1171–1172.