Vol. 88, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1979 Pages 569-574

ACTIVATION OF DICTYOSTELIUM DISCOIDEUM GUANYLATE CYCLASE BY ATP

José M. MATO*

Biozentrum der Universität Basel,
Klingelbergstrasse 70,
CH - 4056 BASEL, Switzerland

Received March 8, 1979

SUMMARY

ATP activates guanylate cyclase in cells of Dictyostelium discoideum. The $K_{0.5}$ for ATP activation is about 50 μM and saturates at about 0.3 mM ATP. Guanylate cyclase activity in crude extracts of D.discoideum shows Michaelian kinetics with a Km of about 1.0 mM GTP and V_{max} of about 30 pmoles cyclic GMP/min/10^6 cells. In the presence of 0.3 mM ATP the enzyme kinetics is also Michaelian, the Km is lowered to about 0.2 mM GTP and the V_{max} remains unchanged. Neither adenylyl-imidodiphosphate nor adenylyl (β,γ-methylene) diphosphonate can substitute ATP as activator of guanylate cyclase. The polyene antibiotic filipin inhibits basal guanylate cyclase activity while ATP activation is not affected.

In aggregating cells of Dictyostelium discoideum cyclic AMP induces chemotaxis (1) and the release of cyclic AMP after a brief stimulation of adenylate cyclase (2). Previously reported results are compatible with a role for cyclic GMP in the processing of a cyclic AMP signal (3-5). Cyclic AMP induces a transient stimulation of guanylate cyclase - EC 4.6.1.2 - (6) which yields a brief increase in the intracellular concentration of cyclic GMP (3,4). Cyclic GMP increase accompanies pseudopod formation (3,4) and precedes cyclic AMP elevation (4). In addition to cyclic AMP, physiological concentrations of ATP also stimulate guanylate cyclase (6). One set of observations led to the conclusion that cyclic AMP and ATP stimulate guanylate cyclase by different mechanisms (6). The studies with ATP suggest a function for this nucleotide in guanylate cyclase regulation in vivo. Therefore we have examined further the mechanism of ATP stimulation of guanylate cyclase in D.discoideum.

* Present address:
Cell Biology and Morphogenesis Unit, Laboratory of Zoology,
Kaiserstraat 63, University of Leiden, 2300 RA Leiden,
The Netherlands.

0006-291X/79/100569-06$01.00/0

METHODS

Organism. Cells of D.discoideum, Ax-2, were grown axenically (7), harvested in the logarithmic growth phase, centrifuged, washed 3 times in cold 17 mM phosphate buffer pH 6.0 and stirred by shaking (8). 5-6 hours after harvesting, cells were washed 3 times in cold phosphate buffer, resuspended at a density of 2×10^8 cells/ml and 2 ml was oxygenated for 20 min.

Guanylate cyclase assay. The oxygenated cell suspension was homogenized by mixing with an equal volume of 100 mM Tris-HCl, pH 7.5, 20% sucrose and 2 mM EDTA and immediate freezing in dry-ice. The frozen cells were then thawed after the addition of ice-cold glycerol at a final concentration of 13%. Guanylate cyclase activity was assayed at 22°C, essentially as in (9), in 250 µl reaction volume containing 50 mM Tris-HCl, pH 7.5, 10 mM dithiothreitol, 0.2 mM GTP, 5 mM $MgCl_2$, 1 mM $MnCl_2$, about 5×10^6 cpm [α-^{32}P] GTP, 20 mM creatine phosphate, 100 units creatine kinase, 13% glycerol and 34 µl homogenate (about 200 µg protein). Reactions were terminated by adding 100 µl assay mixture to 100 µl terminating solution containing 10% SDS, 1.4 mM cyclic GMP, 20 mM GTP and 4000 cpm cyclic [8-^{3}H] GMP.
Cyclic [^{32}P] GMP formed was purified as in (9) by sequential chromatography on Dowex AG50W x 8 and neutral alumina. Recovery ranged from 45-55%.

Materials. [α-^{32}P] GTP (specific activity 10.8 Ci/mmol) and cyclic [8-^{3}H] GMP (specific activity 21 Ci/mmol) were from Amersham. Adenylyl-imidodiphosphate, adenylyl (β,γ-methylene)-diphosphonate GTP, creatin phosphate, creatine kinase and cyclic GMP were from Boehringer. Dithiothreitol was from Sigma and Dowex AG50W x 8 (200 - 400 mesh) and Alumina were from Serva. Filipin U-5956 Crystalline complex 96%, 8393-DEG-11-8 was from the Upjohn Company, Kalamazoo, Michigan, U.S.A.

RESULTS

Guanylate cyclase activity was about 2 fold higher in the presence of 10 mM dithiothreitol, a phosphodiesterase inhibitor in D.discoideum (10), than when 10 mM cyclic GMP was used to protect the cyclic [^{32}P] GMP formed. The addition of 13% glycerol to the assay mixture also enhanced 2 fold guanylate cyclase activity. Therefore 10 mM dithiothreitol and 13% glycerol were used throughout all experiments. In addition to the activation of guanylate cyclase, 13% glycerol protected the enzyme from inactivation. Thus, while in the absence of 13% glycerol complete loss of activity was observed after 1 hour incubation at 4°C in its presence 70-80% of the activity was retained.

Addition of 0.3 mM ATP to the assay mixture activated guanylate cyclase activity 2 fold but could not be mimicked by adenylyl-imidodiphosphate or adenylyl (β,γ-methylene)-diphosphonate even at 1 mM (Fig.1). As shown in Fig.2, guanylate cyclase activity was dependent on the concentration of the added ATP. Half-maximal guanylate cyclase activation occurred at about 50 µM ATP. Concen-

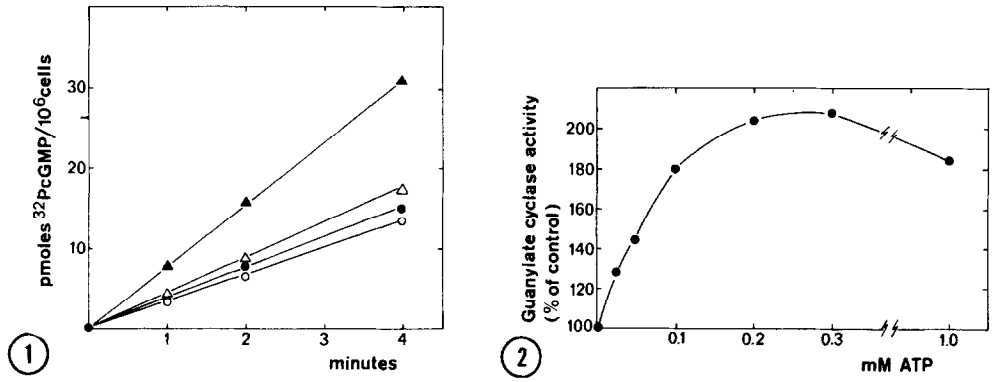


Fig. 1. Effect of ATP, adenylyl-iminodiphosphate and adenylyl (β, γ-methylene)-diphosphonate on guanylate cyclase from D.discoideum. Guanylate cyclase was assayed as described in Methods in the presence of the following additives: none (●); 0.3 mM ATP (▲); 1 mM adenylyl-iminodiphosphate (Δ); and 1 mM adenylyl (β,γ-methylene)-diphosphonate (0).

Fig. 2. Guanylate cyclase activity as a function of the concentration of ATP in the assay mixture. Reactions were terminated at 2 and 4 min and the rate of cyclic (^{32}P)GMP formation calculated. The reactions were linear with time. Values represent the mean of two determinations.

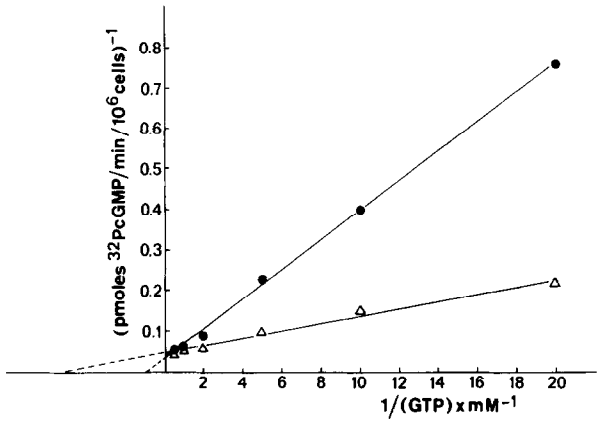
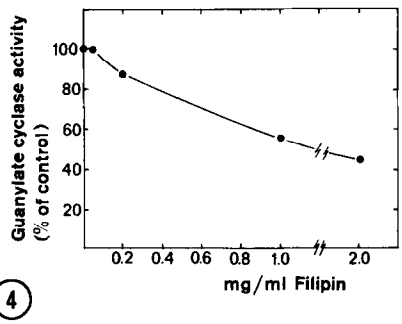


Fig. 3. Double-reciprocal plots of guanylate cyclase activity in the presence or absence of 0.3 mM ATP. Guanylate cyclase activity was measured in the standard assay at different concentrations of GTP in the absence (●) or presence (Δ) of 0.3 mM ATP. At each concentration samples were taken at 2.5 and 5 min and the rate of cyclic (^{32}P)GMP formation calculated. Reactions were linear with time. Values represent the mean of two determinations.

trations of 2 to 3 mM ATP were inhibitory. Guanylate cyclase activity showed Michaelian kinetics with a Km of about 1 mM GTP and V_{max} of 30 pmoles cyclic [^{32}P] GMP/min/10^6 cells (Fig.3). In the presence of 0.3 mM ATP the enzyme kinetics were linear, the Km was lowered to 0.2 mM GTP and the V_{max} remained unchanged (Fig.3).



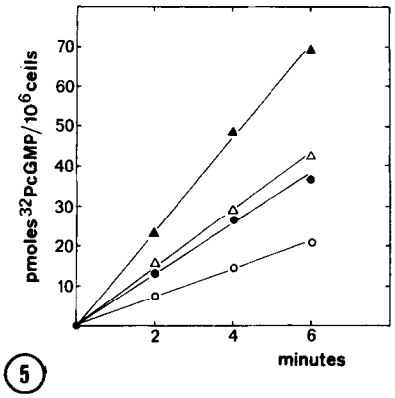


Fig. 4. Guanylate cyclase inhibition by the polyene antibiotic filipin. Prior to the initiation of the reaction, samples were incubated for 5 min at 4°C with filipin or 10% dimethylsulfoxide - the solvent of filipin -. Guanylate cyclase was measured in the standard assay and samples taken at 2 and 4 min. Reactions were linear with time. Values represent the average of two determinations.

Fig. 5. Effect of pre-incubation with filipin on basal and ATP activated guanylate cyclase. Samples were pre-incubated for 5 min at 4°C in the presence of 1 mg/ml filipin (Δ,○) or 10% dimethylsulfoxide (▲,●). Guanylate cyclase activity was then measured in the absence (○,●) or presence (Δ,▲) of 0.3 mM ATP.

Preincubation of cell homogenates of D.discoideum for 5 min at 4°C with the polyene antibiotic filipin inhibited basal guanylate cyclase activity (Fig.4). At a filipin concentration of 1 mg/ml the basal guanylate cyclase activity was decreased about 2 fold while ATP activation was unaffected (Fig.5). After centrifugation for 1 min at 7000 x g guanylate cyclase activity occurs in the pellet and the supernatant (6). Filipin inhibited guanylate cyclase activity from both fractions at about the same magnitude (not shown).

DISCUSSION

Guanylate cyclase activity in aggregating cells of D.discoideum shows Michaelian kinetics with a Km of 1.0 mM GTP. This value lies close to that of 0.5 mM GTP reported in (11) for D.discoideum V-12.

ATP at physiological concentrations increases guanylate cyclase activity by lowering the Km from 1 to 0.2 mM GTP. The activation of the enzyme by ATP might be of physiological importance considering that the mean intracellular GTP concentration in cells of D.discoideum is 0.2 mM (11). The results with

the non-hydrolyzable ATP derivatives suggest a mechanism of activation via phosphorylation. However, if the mechanism of activation is binding and the recognition of ATP involves binding of the γ-oxygen, the substitution of this atom by a nitrogen or methylene will block guanylate cyclase activation.

Guanylate cyclase activation by cyclic AMP and the activation by ATP are additive (6). Cyclic AMP activation is observed only if intact cells are first stimulated, disrupted and guanylate cyclase is then assayed (6). Addition of cyclic AMP to a cell homogenate does not change the rate of cGMP formation (6,9,11). It is therefore not known whether activation of the enzyme by ATP is necessary during in vivo stimulation of guanylate cyclase by cyclic AMP. When a suspension of sensitive cells of D.discoideum is repeatedly stimulated by cyclic AMP within 30 sec cyclic GMP elevation only occurs in response to the first stimulus indicating that desensitization of attractant mediated guanylate cyclase stimulation exists (3). Whether desensitization in vivo occurs at the level of the guanylate cyclase and ATP plays a role is not yet known.

We have previously studied the effect of Triton X-100 on guanylate cyclase from D.discoideum (6). At a concentration of 0.2%, Triton X-100 inhibits about 30% guanylate cyclase activity. After centrifugation at 9000 x g for 1 min, guanylate cyclase of both, pellet and soluble, is inhibited by Triton X-100. These results suggest that certain lipids may surround guanylate cyclase. The results with filipin further suggest that cholesterol interacts with the enzyme and that both fractions, pellet and soluble, are structurally similar.

ACKNOWLEDGEMENTS

I want to thank Drs. G. Gerisch, D. Malchow and T.M. Konijn for their comments. During my stay at the Biozentrum, I was a recipient of a short-term EMBO fellowship.

REFERENCES

1. Konijn, T.M., Barkley, D.S., Chang, Y.Y.,and Bonner, J.T. (1968) Am Naturalist 102, 225-234.
2. Roos, W.,and Gerisch, G. (1976) FEBS Lett. 68, 170-172.
3. Mato, J.M., Krens, F.A., van Haastert, P.J.M.,and Konijn, T.M. (1977) Proc. Natl. Acad. Sci. USA 74, 2348-2351.

4. Wurster, B., Schubiger, K., Wick, U., and Gerisch, G. (1977) FEBS Lett. 76, 141-144.
5. Mato, J.M., van Haastert, P.J.M., Krens, F.A., Rhijnsburger, E.H., Dobbe, F.C.P.M., and Konijn, T.M. (1977) FEBS Lett. 79, 331-336.
6. Mato, J.M., and Malchow, D. (1978) FEBS Lett. 90, 119-122.
7. Watts, D.J., and Ashworth, J.M. (1970) Biochem. J. 119, 171-174.
8. Gerisch, G. (1962) Wilhelm Roux Arch. Entwicklungsmech. 153, 603-620.
9. Mato, J.M., Roos, W., and Wurster, B. (1978) Differentiation 10, 129-132.
10. Chassy, B.M. (1972) Science 175, 1016-1018.
11. Ward, A., and Brenner, M. (1972) Life Sci. 21, 997-1008.