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A MAGNESIUM-DEPENDENT GUANYLATE CYCLASE IN CELL-FREE PREPARATIONS OF DICTYOSTELIUM DISCOIDEUM

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Summary. Receptor-mediated regulation of guanylate cyclase is well-studied in intact *Dictyostelium discoideum* cells, but study of the enzyme in cell-free preparations has hampered. A major obstacle has been that *in vitro* guanylate cyclase activity could be detected only in the presence of unphysiological concentrations of Mn^{2+}-ions. In this paper we report the identification of a guanylate cyclase in *D.discoideum* cell homogenates that has high activity with Mg^{2+}-GTP. The enzyme is activated by non-hydrolyzable ATP and GTP analogues and inhibited by submicromolar concentrations of Ca^{2+}-ions. We suggest that the presently identified enzyme is regulated in intact cells via cell surface receptors. The compounds that modulated the enzyme activity *in vitro* may reflect physiologically relevant regulation mechanisms.

Receptors for chemoattractants on the surface of the slime mold *Dictyostelium discoideum* regulate a host of cellular responses, among others the activity of guanylate cyclase. In vegetative *D.discoideum* cells guanylate cyclase is regulated by receptors for folic acid, in starving cells by receptors for cAMP (1). Slime mold guanylate cyclase has recently attracted attention, firstly because evidence has been found suggesting that its activity is regulated via the phos-

Abbreviations. App[NH]p, adenosine 5'-[β,γ-imido] triphosphate, ATPγS, adenosine 5'O-(thio)triphosphate, Ins(1,4,5)P_3, D-myo-inositol 1,4,5-trisphosphate, GTPγS, guanosine 5'O-(thio)triphosphate, EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid, EDTA, ethylene diamino tetraacetic acid, Hepes, N-(2-hydroxyethyl)piperazine-N'2-ethanesulfonic acid.

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phatidylinositol cycle (2,3), and secondly because oncogenic *ras* proteins may play a role in its desensitization (4).

Despite the extensive studies on *Dictyostelium* guanylate cyclase with intact and permeabilized cells, little progress has been made with study of the enzyme in cell-free preparations. A major obstacle for *in vitro* studies has been the lack of guanylate cyclase activity with Mg^{2+}-GTP. Unphysiological, millimolar concentrations of Mn^{2+}-ions were required for activity (5,6). We now have identified a Mg^{2+}-dependent enzyme and describe the conditions required for its detection.

Materials and Methods

D.discoideum NC4(H) was grown, harvested and freed from bacteria (7). Cells were starved on non-nutrient agar at 6 °C for 16-20 h, harvested, washed and resuspended at 10^8 cells/ml in lysis buffer (40 mM Hepes\NaOH, 3 mM $MgSO_4$, 1 mM EGTA, 10^{-4} M App[NH]p, pH 7.0). Homogenization was performed by rapid elution of cell suspensions through polycarbonate filters (Nuclepore, Pleasanton CA, pore size 3 µm), (8).

Guanylate cyclase activity was measured as described before (9,10), by adding homogenate to a reaction mixture containing 0.6 mM GTP and 10 mM dithiothreitol, giving the following concentrations during the reaction (25 °C): 20 mM Hepes\NaOH, 1.5 mM $MgSO_4$, 0.5 mM EGTA, 5×10^{-5} M App[NH]p, 0.3 mM GTP, 5 mM dithiothreitol, pH 7.0. $MnCl_2$, if added, was present at 5 mM. Reactions were started 50 s after cell lysis, unless otherwise indicated.

Results

Our approach to obtain Mg^{2+}-dependent guanylate cyclase was basically the same as the one recently described for detecting guanine nucleotide-sensitive adenylate cyclase in *Dictyostelium* (8). Cells were lysed by polycarbonate filtration at 0 °C and within 1 min after cell lysis homogenates were incubated at 25 °C in the presence of GTP and a phosphodiesterase inhibitor . Using this approach, cGMP was produced in the absence of exogenous Mn^{2+}-ions (fig.1). cGMP was not produced by intact cells incubated in this way. The cGMP synthesis by homogenates using Mg^{2+}-GTP was linear for about

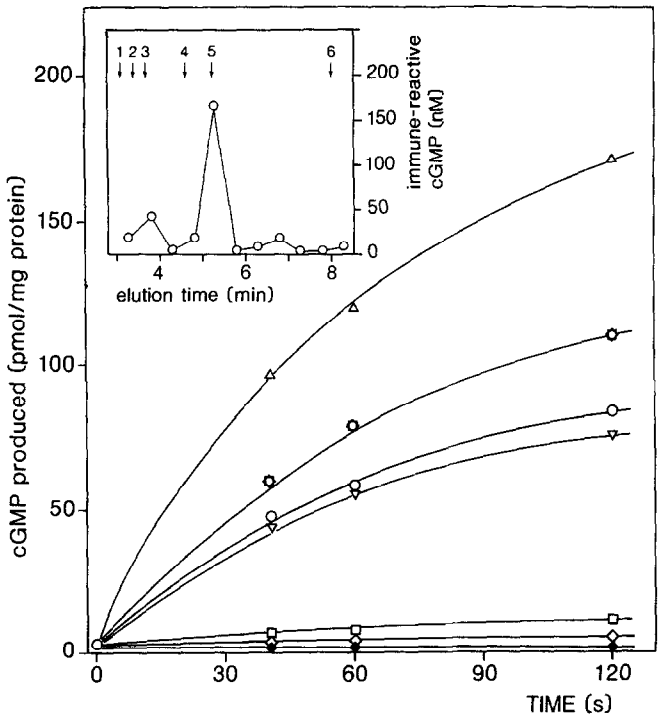

Fig.1. cGMP production by preparations obtained from *D.discoideum*. Cell homogenates or intact cells were transferred at t=0 s to various reaction media (25°C). Samples were taken at different times to be analyzed for cGMP content by radioimmunoassay(10). The reaction conditions were either as described in Materials and Methods (○,◆), or modified as follows: (○) 2 mM GTP; (▽) $MnCl_2$, 0.3 mM GTP; (△) $MnCl_2$, 2 mM GTP; (◇) no GTP; (□) 4 mM EDTA. Intact cells: (◆); others: homogenates. Inset: analysis of reaction products by HPLC on Lichrosorb 10 RP 18, as described (11). Homogenate was incubated 1 min with reaction medium containing GTP, Mg^{2+} and dithiothreitol and transferred to $HClO_4$. The sample was neutralized and eluted with 20% methanol, 1 mM phosphate buffer pH 6.5. Fractions (0.5 ml) were lyophylized and their cGMP content was measured by radioimmunoassay. The recovery of cGMP in the sample was 67%. The elution positions of 1) 5'GMP, 2) GDP, 3) GTP, App[NH]p, 4) cIMP, 5) cGMP and 6) cAMP are indicated.

1 min and was about as high as in the presence of Mn^{2+}-ions (fig.1). Similar activity was measured with 0.75-7.5 mM Mg^{2+}-ions (not shown); the cGMP synthesis was strongly reduced under nominally free Mg^{2+}- and Mn^{2+}-conditions (excess EDTA), or when GTP was left from the reaction mixture (fig.1). Replacement of GTP by ATP did not result in cGMP production any higher than in the absence of GTP (not shown). The product synthesized by homogenates incubated with Mg^{2+}-GTP was also identified as cGMP by HPLC (fig.1, inset). From these results we conclude that we were measuring a Mg^{2+}-dependent guanylate cyclase in *Dictyostelium* cell homogenates.

Table I. Factors affecting the activity of Mg^{2+}-dependent guanylate cyclase in homogenates of *D. discoideum* cells

Lysis condition additions to/omissions from lysis buffer	Guanylate cyclase pmol/min.mg protein ±SEM (n)
1. none	66 ± 12 (6)
2. sucrose	29 ± 12 (4)
3. glycerol	25 ± 9 (3)
4. no App[NH]p	12 (2)
5. App[NH]p replaced by ATP	19 ± 1 (3)
6. App[NH]p replaced by ATPγS	18 ± 1 (3)
7. GTPγS	188 ± 43 (7)
8. GTPγS; no App[NH]p	98 ± 7 (8)
9. no EGTA	26 ± 14 (4)
10. lysis by freeze thawing	< 5 (2)

In all cases, except 10), cells were lysed by elution through polycarbonate filters and in lysis buffer as described in Materials and Methods. ATP, ATPγS and GTPγS, if indicated, were present at a concentration of 25 μM, sucrose and glycerol at 250 mM and 1.78 M, respectively.

The activity of Mg^{2+}-dependent guanylate cyclase was about 2-fold less when sucrose or glycerol were present in lysis buffer (table I). Both compounds have frequently been used to stabilize Mn^{2+}-dependent guanylate cyclase (5,6,10, 12). The ATP analogue App[NH]p activated Mg^{2+}-dependent guanylate cyclase (2 to 5-fold), like it does the Mn^{2+}-dependent enzyme (table I), (6,12,13). In contrast, Mg^{2+}-dependent guanylate cyclase was not activated by ATP (table I). The GTP analogue GTPγS also stimulated guanylate cyclase but its adenine analogue, ATPγS, did not activate the enzyme (table I). This suggests that the stimulation by GTPγS and App[NH]p are not mechanistically related. Addition of the Ca^{2+}-chelator EGTA to cell homogenates resulted in two fold higher guanylate cyclase activity (table I). This effect may result from the elimination of Ca^{2+}-ions from homogenates, as Ca^{2+}-ions strongly inhibited Mg^{2+}-dependent cyclase (fig.2). Inhibi-

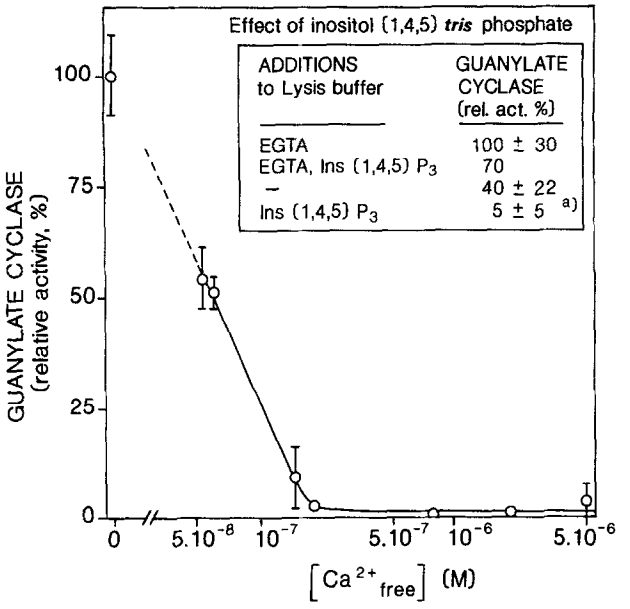

Fig.2. Effect of Ca^{2+}-ions and inositol 1,4,5-trisphosphate on Mg^{2+}-dependent guanylate cyclase. Cell homogenates were prepared in lysis buffer with Ca/EGTA buffers determining the free Ca^{2+}-concentration (14), as indicated. $[Ca^{2+}_{free}] = 0$ M denotes the condition with excess EGTA (5.9 mM) and no exogenous $CaCl_2$. Inset: the effect of 10 µM $Ins(1,4,5)P_3$ during lysis in the absence or presence of EGTA (no exogenous Ca^{2+}), as indicated. Data in the main figure are means ± SEM from three experiments, those in the inset from 2-4 experiments. a) In the absence of EGTA the inhibition of guanylate cyclase by $Ins(1,4,5)P_3$ was significant at $P < 0.1$ (Student's t-test).

tion, definitely not activation, was also found with inositol 1,4,5-trisphosphate [$Ins(1,4,5)P_3$], (fig.2).

Mg^{2+}-dependent guanylate cyclase activity was rapidly lost from cell homogenates ($t_{0.5}$ = 2-3 min). This was not specific for the Mg^{2+}-dependent enzyme, as the activity measured in the presence of Mn^{2+}-ions disappeared equally rapid (fig.3). After cell lysis by freeze-thawing, no guanylate cyclase activity was detected using Mg^{2+}-GTP (table I).

Discussion

We report for the first time on a guanylate cyclase in cell-free preparations of *D.discoideum*, that uses Mg^{2+}-GTP as a substrate. Various factors were found to determine the activity of this enzyme: sucrose, glycerol and Ca^{2+}-ions inhi-

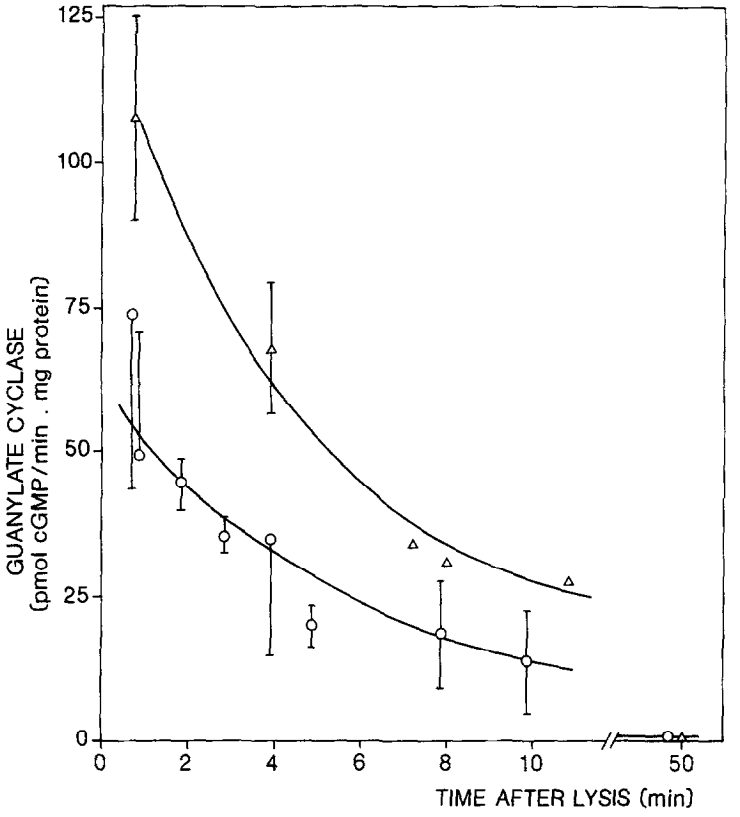

Fig.3. Decay of the guanylate cyclase activity in cell homogenates. Homogenates were prepared at t=0 min, and incubated at 0 °C. After various times samples were assayed for guanylate cyclase activity at 25 °C, in mixtures containing: (○) Mg^{2+}-ions and 0.3 mM GTP, or (△) Mn^{2+}-ions and 2 mM GTP. Data are means (± SEM) from data of 2-4 experiments.

bit, the nucleotides App[NH]p and GTPγS stimulate the enzyme. Furthermore, the method of cell lysis is important, while measurements soon after cell lysis are required because of the rapid loss of the enzyme activity in homogenates.

It is attractive to assume that the Mg^{2+}-dependent guanylate cyclase presently identified is identical to the enzyme that in intact cells is regulated via cell surface receptors. However, this remains to be proven; we have not yet been able to demonstrate effects of receptor ligands on the enzyme. The conditions presently described for measuring guanylate cyclase *in vitro*, reflect the *in vivo* situation more than before. Hence, the factors that were found to affect the activity may be relevant with regard to the regulation of the

enzyme *in vivo*. Notable are Ca^{2+}-ions, Ins(1,4,5)P_3 and the GTP analogue GTPγS. Ca^{2+}-ions and Ins(1,4,5)P_3 evoke a cGMP response in permeabilized *D.discoideum* cells, maybe by stimulating guanylate cyclase (2,3). However, rather than activating, Ca^{2+}-ions and Ins(1,4,5)P_3 inhibited Mg^{2+}-dependent guanylate cyclase. This could mean that their effects in permeabilized cells are indirect. The effect of GTPγS is of interest, because it suggests a novel regulatory mechanism for guanylate cyclase in *Dictyostelium*. This might involve a regulatory component e.g. a G-protein. Some evidence for regulation of guanylate cyclase by G-proteins also exists in vertebrates (15). Further studies are required at this point.

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