Regulatory Properties of Magnesium-dependent Guanylate Cyclase in Dictyostelium discoideum Membranes*

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We have characterized a magnesium-dependent guanylate cyclase in homogenates of Dictyostelium discoideum cells. 1) The enzyme shows an up to 4-fold higher cGMP synthesis in the presence of GTP analogues with half-maximal activation at about 1 μM guanosine 5'-O-(3-thio)triphosphate (GTPyS) or 100 μM guanosine 5'- (β,γ-imido)triphosphate; little or no stimulation was observed with GTP, guanosine mono- and diphosphates or with adenine nucleotides, with the exception of the ATP analogue adenosine 5'- (β,γ-imido)triphosphate. 2) Both basal and GTPyS-stimulated guanylate cyclase activity were rapidly lost from homogenates as was the ability of GTPyS to stimulate the enzyme after cell lysis. 3) Inclusion of 25 μM GTPyS during cell lysis reduced the KM for GTP from 340 to 85 μM and increased the Vmax from 120 to 255 pmol/min/mg protein, as assayed in homogenates 90 s after cell lysis. 4) Besides acting as an activator, GTPyS was also a substrate for the enzyme with a KM = 120 μM and a Vmax = 115 pmol/min/mg protein. 5) GTPyS-stimulated, Mg2+-dependent guanylate cyclase was inhibited by submicromolar concentrations of Ca2+ ions, and by inositol 1,4,5-trisphosphate in the absence of Ca2+ chelators. 6) Guanylate cyclase activity was detected in both supernatant and pellet fractions after 1 min centrifugation at 10,000 x g; however, only sedimentable enzyme was stimulated by GTPyS. We suggest that the Mg2+-dependent guanylate cyclase identified represents the enzyme that in intact cells is regulated via cell surface receptors, and we propose that guanine nucleotides are allosteric activators of this enzyme and that Ca2+ ions play a role in the maintenance of the enzyme in its basal state.

In the cellular slime mold Dictyostelium discoideum, intracellular guanylate cyclase is activated up to 10-fold by chemotaxants that bind to cell surface receptors. Other well studied responses regulated via receptors are the activation of adenylate cyclase, the phosphorylation of chemotactic cAMP receptors, and alterations in the polymerization state of actin (1). Signal transduction in D. discoideum shows remarkable similarities with that in higher eukaryotes that bind to cell surface receptors, and the regulation of adenylate cyclase by GTP-binding proteins, the mechanism of desensitization, and the likely presence of a phosphatidylinositol cycle (2). On the other hand, mechanisms have been proposed to operate in Dictyostelium which have no precedent in other organisms. Examples are an involvement of the phosphatidylinositol cycle in regulation of guanylate cyclase (3, 4), and the role that ras proteins might have in the desensitization of this enzyme (5). The latter findings have attracted attention, also because limited insight exists into the functioning of receptor-regulated guanylate cyclases in eukaryotes in general (cf. Ref. 6).

Despite extensive studies on the activation and desensitization of guanylate cyclase in intact D. discoideum cells (see Ref. 1), little is known about the direct regulation of the enzyme. There exists only one report on the modulation of guanylate cyclase activity in cell-free preparations via cell surface receptors: when cells were stimulated with cAMP and rapidly lysed, the enzyme was found to be stimulated 3-6-fold (7). In vitro, chemicals like adenosine triphosphates and glycerol modulate the activity of guanylate cyclase (8-10). However, in all these investigations (7-10) millimolar concentrations of Mn2+ ions have been used in the enzyme assays, because with Mg2+-GTP little guanylate cyclase activity was found (9, 10). As the intracellular Mn2+ concentrations in Dictyostelium are in the micromolar range (9) the relevance of these studies for insight into the regulation of guanylate cyclase may be called into question. Permeabilized cells have been an alternative tool to the study of Dictyostelium guanylate cyclase. It was described that the elevation of the cGMP concentration evoked by chemoattractants was mimicked by addition of Ca2+ ions or inositol 1,4,5-trisphosphate to permeabilized cells (3, 4). Permeabilized cells, however, offer limited possibilities for manipulation of the enzyme and control of its environment.

We have recently managed to identify a guanylate cyclase in cell-free preparations that is active with Mg2+-GTP (11). This enzyme was observed in homogenates, immediately after cell lysis. In this report we describe some salient features of this enzyme, which is present in membranes, has increased activity in the presence of guanosine triphosphates, and is inhibited by Ca2+ ions.

**EXPERIMENTAL PROCEDURES**

Culture Conditions and Cell Lysis—D. discoideum NC4(H) cells were grown, harvested in the late logarithmic phase with 10 mM Na+/K+-phosphate buffer, pH 6.5, and freed from bacteria by centrifugation at 100 x g for 4 min (12). Cells were developed until aggregation competence by starvation on non-nutrient agar at 6 °C for 16-20 h; subsequently they were harvested, washed, and resuspended at a
density of 10^6 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, unless otherwise indicated). Homogenization was performed at 0°C by rapid elution of cell suspensions through polycarbonate filters (pore size 3 μm), by a method developed before for lysis of small cell volumes (100-300 μl) (13, 14). This method resulted in >99% cell lysis, as estimated by phase contrast microscopy.

**Guanylate Cyclase Assay**—Guanylate cyclase activity was measured at 25°C using the method briefly described before (11). Standard reaction mixture contained 20 mM Hepes/NaOH, 1.5 mM MgSO_4, 0.5 mM EGTA, 5 X 10^{-5} M App[NH]p, 5 mM dithiothreitol, and 0.3 mM GTP (final concentrations), pH 7.0. For investigation of the enzyme kinetics the GTP or GTPγS concentrations were varied, while keeping the Mg^{2+} concentration at 1.2 mM in excess to the guanine nucleotides by adding extra MgCl_2. MnCl_2, if added, was present at a concentration of 5 mM. Reactions were started between 40 and 120 s after cell lysis, unless otherwise indicated; samples were routinely taken at 0, 40, and 60 s after initiation of the reaction and quenched in 4 N HClO_4. Samples were processed, and cGMP produced was measured in a radioimmunoassay (15, 16). Cyclic GMP synthesis was linear up to about 1.5 mg of protein/ml in the guanylate cyclase assay. Cyclic AMP was measured according to the method of Tovey et al. (17).

**High Performance Liquid Chromatography Analysis**—Reaction products formed during the guanylate cyclase reaction were analyzed by high performance liquid chromatograph on Lichrosorb RP-18. Samples from the cyclase reaction in HClO_4 were neutralized with K_2CO_3, adjusted to pH 7.0, and either treated with phosphodiesterase or directly applied to the high performance liquid chromatography column. Phosphodiesterase treatment was done for 1 h at 22°C with 0.1 mg/ml beef heart phosphodiesterase in the presence of 4 mM MgCl_2, the reaction was arrested by 3 min heating at 99°C. The high performance liquid chromatography column was eluted with 20% methanol, 1 mM K+ phosphate, pH 6.5, at 1 ml/min. Fractions were collected, lyophilized, and their cGMP content was measured by radioimmunoassay (15, 16).

**Materials**—GTP, cGMP, ATP, and dithiothreitol were obtained from Sigma; GTPγS, Gpp[NH]p, GDDPγS, APP[NH]p, and beef heart phosphodiesterase were from Boehringer (Mannheim, Federal Republic of Germany); [8-^3H]cGMP (15 Ci/mmol) and inositol 1,4,5-trisphosphate were from Amersham International (Amersham, United Kingdom); Lichrosorb 10 PR18 was from Chrompack (Middleburg, The Netherlands), and polycarbonate filters were from Nucleopore (Pleasanton, CA).

**RESULTS**

**Increased Mg^{2+}-dependent Guanylate Cyclase Activity in the Presence of GTPγS**

For obtaining Mg^{2+}-dependent guanylate cyclase, cells in a buffer containing Mg^{2+} ions and various additions were lysed by elution through polycarbonate filters, and shortly after lysis homogenates were transferred to a reaction mixture, as described before (11).

When the GTP analogue GTPγS (25 μM) was present during lysis, a marked, about 3-fold higher cGMP production was observed (Table I). It was indeed the activity of a guanylate cyclase that was detected here under cell-free conditions: no cGMP production was obtained with intact cells, and omission of GTP from the reaction mixture resulted in a strong reduction of the cGMP synthesis (Table I), (11). When GTP was replaced by ATP in the reaction mixture a similar reduction of the cGMP production was obtained, while concurrently the synthesis of cAMP was stimulated strongly (Table I). Although the elimination of GTP from reaction mixtures resulted in reduced cGMP production, this produc-

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1The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine 5'-O-(3-thio)triphosphate; Gpp[NH]p, guanosine 5'-O-(2-thio)diphosphate; GDDPγS, guanosine 5'-O-(2-thio)diphosphate; Gpp[NH]p, guanosine 5'-O-(2-thio)diphosphate; GMP, guanosine 5'-O-(2-thio)diphosphate; GTP, guanosine 5'-O-(3-thio)triphosphate; cGMP, cyclic GMP; cAMP, cyclic AMP; KOH/KHCO_3 (15) and either treated with phosphodiesterase or high performance liquid chromatography on Lichrosorb RP-18. Reaction products formed during the guanylate cyclase reaction were analyzed by high performance liquid chromatography on Lichrosorb RP-18. Samples from the cyclase reaction in HClO_4 were neutralized with K_2CO_3, adjusted to pH 7.0, and either treated with phosphodiesterase or directly applied to the high performance liquid chromatography column. Phosphodiesterase treatment was done for 1 h at 22°C with 0.1 mg/ml beef heart phosphodiesterase in the presence of 4 mM MgCl_2; the reaction was arrested by 3 min heating at 99°C. The high performance liquid chromatography column was eluted with 20% methanol, 1 mM K+ phosphate, pH 6.5, at 1 ml/min. Fractions were collected, lyophilized, and their cGMP content was measured by radioimmunoassay (15, 16).

**Factors Affecting the Activity of Mg^{2+}-dependent Guanylate Cyclase in the Presence of GTPγS**

**Time after Cell Lysis**—Cell homogenates were prepared in the presence or absence of GTPγS (25 μM) and the activity of Mg^{2+}-dependent guanylate cyclase was measured at different times after cell lysis (Fig. 1). Under both conditions the enzyme activity was rapidly lost. In homogenates prepared in the absence of GTPγS no guanylate cyclase activity was detected at 50 min after cell lysis, whereas in homogenates prepared in the presence of GTPγS the activity was decreased to 10% of the activity directly after cell lysis. The loss of Mg^{2+}-dependent guanylate cyclase activity in homogenates prepared in the presence of GTPγS showed characteristics of a first order exponential decay, described by a k_1 = 0.115 min^{-1} (τ_0.5 = 6 min), (Fig. 1, inset). The decay of guanylate cyclase activity in homogenates not containing GTPγS appeared to be multiphasic (Fig. 1, inset). Shortly after cell lysis it oc-

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**Table I**

| Additon/condition | Cyclic nucleotide production |
|-------------------|-----------------------------|
| During lysis     | During enzyme reaction      | cGMP | cAMP |
| Standard         | Standard                    |      |      |
| GTPγS, No cell lysis | Standard                  |      |      |
| GTPγS, ro cell lysis | Standard                   |      |      |

*Standard conditions involved cell lysis in 40 mM Hepes/NaOH, 3 mM MgSO_4, 1 mM EGTA, 10^{-4} M App[NH]p, pH 7.0 (0°C). Enzyme reactions were done in 20 mM Hepes/NaOH, 1.5 mM MgSO_4, 0.5 mM EGTA, 0.5 X 10^{-5} M App[NH]p, 0.3 mM GTP, and 5 mM dithiothreitol, pH 7.0, or with modifications as indicated. GTPγS, if indicated, was present at 25 μM, EDTA at 4 mM, MnCl_2 at 5 mM, cGMP at 200 or 400 nM, and ATP at 0.3 mM. Cyclic AMP measurements are from one experiment.

**Identification of a GTPγS-stimulated, Mg^{2+}-dependent guanylate cyclase in Dictyostelium cell homogenates**

| Additon/condition | Cyclic nucleotide production |
|-------------------|-----------------------------|
| During lysis     | During enzyme reaction      | cGMP | cAMP |
| Standard         | Standard                    |      |      |
| GTPγS, No cell lysis | Standard                  |      |      |
| GTPγS, ro cell lysis | Standard                   |      |      |

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The effect of GTPγS on cGMP production resulted from stimulation of a guanylate cyclase and not from inhibition of a phosphodiesterase. This was inferred from measurements of the phosphodiesterase activity under the reaction conditions, which were done by replacing GTP by cGMP. The degradation of cGMP was about 15%/min and was not reduced in the presence of GTPγS (Table I). Furthermore, stimulation of cGMP production by GTPγS was also observed in homogenates from a Streamer F mutant of D. discoideum (not shown), which is a strain that lacks cGMP-specific phosphodiesterase (18, 19).
Mg\textsuperscript{2+}-dependent Guanylate Cyclase in D. discoideum

![Graph showing decay of guanylate cyclase activity](image)

**Fig. 1.** Decay of the guanylate cyclase activity in cell homogenates. Homogenates were prepared in the absence of (C, D) or presence (A, B) of 25 μM GTPγS at time = 0 min. After various incubation times at 0 °C samples were assayed for guanylate cyclase activity, either under standard conditions (Mg\textsuperscript{2+}/0.3 mM GTP; O, •) or in standard reaction medium containing 2 mM GTP and 5 mM MnCl\textsubscript{2} (C, □). The inset shows a semilogarithmic plot of the data from reactions not containing Mn\textsuperscript{2+} ions. Data are the means ± S.E. of 2–4 experiments.

**Fig. 2.** Effect of addition of GTPγS after cell lysis on the activity of guanylate cyclase. D. discoideum cells were lysed at time = 0 s and at different times after lysis 25 μM GTPγS was added to homogenates (0°C) (filled symbols). Once 25 μM GTPγS was added prior to lysis (filled symbols at t = 0 s); in two cases homogenates encountered GTPγS comitantly with reaction mixture (filled symbols at 48 and 164 s). Open symbols, guanylate cyclase activities from homogenates that received no GTPγS. For each preparation, the guanylate cyclase activity was assayed at 48 s (□, •) and 164 s (△, △) after cell lysis. Data are the means ± S.E. of measurements from 5 different experiments.

**Fig. 3.** Effect of Ca\textsuperscript{2+} ions and inositol 1,4,5-trisphosphate on basal and GTPγS-stimulated, Mg\textsuperscript{2+}-dependent guanylate cyclase. D. discoideum cell homogenates were prepared in lysis buffer in the absence or presence of 25 μM GTPγS (open symbols or hatched bars, and filled symbols or open bars, respectively). a, the free Ca\textsuperscript{2+} concentration during lysis was varied by adding 1–6 mM CaCl\textsubscript{2} to lysis buffer and 5.9 mM EGTA. [Ca\textsuperscript{2+} free] = 0 μM denotes the condition in which 5.9 mM EGTA was present and CaCl\textsubscript{2} was omitted. The K\textsubscript{d} for the Ca + EGTA = Ca-EGTA equilibrium at pH 7.0 was taken 1.85 × 10\textsuperscript{-7} M (20). The free Ca\textsuperscript{2+} concentrations were calculated using log([Ca\textsuperscript{2+} free]) = log K\textsubscript{d} + log ([Ca-EGTA]/[EGTA-free]). Data are the means ± S.E. from 3 separate experiments. b, guanylate cyclase activity in homogenates prepared in the absence or presence of 5 mM EGTA or 10 μM inositol 1,4,5-trisphosphate (Ins(1,4,5)P\textsubscript{3}), as indicated. Data are the means ± S.E. of 2–7 lysates. * denotes the difference is significant at p < 0.1; **, the difference is not significant at p > 0.4 (Student’s t test, non-paired).

![Graph showing effect of Ca\textsuperscript{2+} ions and inositol 1,4,5-trisphosphate](image)
lision buffer resulted in about 50% lower guanylate cyclase activity than in the presence of EGTA, both in the presence and absence of GTPyS (11; and data not shown). Considering the strong inhibition of Mg2+-dependent guanylate cyclase by micromolar concentrations of Ca2+ ions, and the specificity of EGTA for Ca2+ ions (20), the higher activity in the presence of EGTA can probably be attributed to the elimination from the homogenate of free Ca2+ ions originating from cells.

Ins(1,4,5)P3, like Ca2+ ions, did not stimulate basal or GTPyS-stimulated guanylate cyclase (Fig. 3b). Rather, in the absence of EGTA, inhibition of guanylate cyclase was observed (significance p < 0.1). This may be explained by an Ins(1,4,5)P3-mediated release of Ca2+ ions from intracellular stores present in the homogenates. Such a release has been demonstrated in permeabilized D. discoideum cells (21). Considering the sensitivity of Mg2+-dependent guanylate cyclase to Ca2+ ions (Fig. 3a), the Ca2+ release induced by Ins(1,4,5)P3 would be predicted to be about 40 nM. The above explanation agrees with the observation that Ins(1,4,5)P3 did not inhibit guanylate cyclase in the presence of EGTA (Fig. 5b).

Ca2+ ions (2 or 100 μM) also strongly inhibited Mg2+-
dependent guanylate cyclase in homogenates, prepared from cells that had been treated with saponin (1 mg/ml, 30 min). The Mg2+-dependent enzyme was also inhibited by Ca2+ ions when saponin (1 mg/ml) was added to homogenates immediately after cell lysis and prior to the guanylate cyclase assay (data not shown). Thus, the difference in the effects of Ca2+ on cGMP production in permeabilized cells (3, 4) and in cell homogenates does not result from the use of saponin in the former system. When we tried to reproduce the previous findings with saponin-permeabilized cells (3, 4), we were able to induce a slight cGMP accumulation using Ins(1,4,5)P3 (maximally 10–25% of the accumulation induced by chemottractants); however, we were unable to induce a cGMP response by use of Ca2+ ions.2

Effect of pH and Adenine Nucleotides— Guanylate cyclase in homogenates prepared both in the absence and presence of GTPyS had optimal activity between pH 7.5 and 8.0 (not shown). This is the same pH optimum as reported for Mn2+-dependent guanylate cyclase in Dictyostelium (10). Below pH 7.0 almost no cyclase activity was detectable. Above pH 7.0 the cyclase activity in homogenates not containing GTPyS was also less than about 50% of the activity in the presence of GTPyS. Hence, no evidence was found for pH-dependent uncoupling of guanylate cyclase from its GTPyS sensitivity.

The effect of adenine nucleotides on the GTPyS-stimulated guanylate cyclase was the same as the effect on the enzyme in the absence of GTPyS, described before (11). Thus, App(NH)p stimulated guanylate cyclase in the presence of GTPyS the same amount (2–3-fold) as in its absence, whereas ATP and ATPyS had no effect on the basal or GTPyS-stimulated enzyme activity (11, and data not shown). As noted before (11), the latter observation suggests that the stimulation of the enzyme by guanine nucleotides (see below) is not mechanistically related to that by adenine nucleotides.

Characterization of the Effect of Guanine Nucleotides on Mg2+-dependent Guanylate Cyclase

Dose response curves of the effect of guanine nucleotides on the activity of Mg2+-dependent guanylate cyclase are shown in Fig. 4. The most potent guanine nucleotide was GTPyS, which stimulated half-maximally at about 1 μM. Maximal, about 4-fold, stimulation was reached at about 10-4 M GTPyS. A similar -fold amount of stimulation was observed with Mg2+-dependent guanylate cyclase from vegetative and aggregation-competent Dictyostelium cells. The GTP analogue Gpp(NH)p stimulated guanylate cyclase up to about 2-fold at concentrations around 10-4 M. Inclusion of GTP at concentrations of about 10-4 M during cell lysis also resulted in a small increase in the cGMP production by homogenates (Fig. 4). This effect, however, may be explained by an increase in the substrate concentration for guanylate cyclase, taking into account the Km of the enzyme for GTP (see below) and the subsaturating GTP concentration normally used in the enzyme reactions. Therefore, in some experiments, the GTP concentration in the reaction mixtures was adjusted so that homogenates prepared in the presence of GTP were assayed at the same GTP concentration as controls from homogenates that had not received GTP during lysis. In these experiments the presence of 4 mM GTP during cell lysis resulted in only 40% more cGMP synthesis as compared to the synthesis by lysates prepared in the absence of GTP.

Guanosine diphosphates, like GDP and GDPβS, and guanosine monophosphate (GMP) did not activate guanylate cyclase (Fig. 4). Rather, they tended to inhibit the enzyme. GDPβS also antagonized the activation by GTPyS, when present in excess of this compound (Fig. 4, inset). This might be explained by competition of GDPβS with GTPyS for a regulatory site or by competition of GDPβS with GTP or GTPγS at the catalytic site of the enzyme. The latter explanation is favored by the observation that GDPβS inhibited the cGMP synthesis in the presence and absence of GTPyS to relatively the same amount (about 50% at 100 μM GDPβS, Fig. 4, inset).

The activation of Dictyostelium guanylate cyclase by guanosine triphosphates has some characteristics similar to the activation of Dictyostelium adenylate cyclase, with regard to its nucleotide specificity and the property to give highest activity shortly after cell lysis (13). A difference, however, is

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2 P. J. M. Van Haastert and R. J. W. DeWit, unpublished observations.
that guanylate cyclase activity decreased rather than increased during incubation at 0 °C in the presence of GTPyS (Figs. 1 and 2), while for adenylate cyclase such an incubation is required to obtain maximal activity (13). Another difference between both enzymes was observed when studying the aggregation-less mutant synag 7 (also called N7). Activation of membrane-bound adenylate cyclase requires the presence of a soluble cytosolic factor and does not occur in this mutant, which has a defect in this factor (13, 22). As shown in Table II, homogenates of mutant synag 7 contained about 5-fold less guanylate cyclase activity than wild type homogenates, as measured with Mn2+-GTP as a substrate. Basal Mg2+-dependent guanylate cyclase activity could not be detected. However, when GTPyS was included in homogenates a Mg2+-dependent guanylate cyclase activity, comparable to the activity in the presence of Mn2+ ions, was apparent. Thus, in contrast to adenylate cyclase in mutant synag 7 (13, 22), guanylate cyclase showed no defect in its activation by GTPyS (Table II). These observations suggest that, despite some superficial resemblances, Dictyostelium guanylate cyclase and adenylate cyclase are activated by guanine nucleotides by different mechanisms.

Kinetics of GTPyS-stimulated, Mg2+-dependent Guanylate Cyclase

The kinetics of Mg2+-dependent guanylate cyclase were studied under three different conditions. Either homogenates were prepared in the absence of exogenously added guanine nucleotides and the activity of the enzyme was measured with various concentrations of GTP or GTPyS; or homogenates were prepared in the presence of a low concentration of GTPyS and the enzyme activity was determined with various concentrations of GTP during the reaction. These experiments might shed light on the mechanism of activation of the enzyme by GTPyS and answer the question whether GTPyS was (also) a substrate for the enzyme.

The results of these kinetic measurements are compiled in Table III. Under each condition tested, the behavior of guanylate cyclase conformed to Michaelis-Menten kinetics. Relatively, the lowest activity was detected with the enzyme prepared in the absence of GTPyS, using GTP as substrate (Table III, part I). Guanylate cyclase also effectively used GTPyS as a substrate, showing the same Vmax and about a 3-fold lower Km with GTPyS than with GTP (Table III, part II). When GTPyS was present during cell lysis and the enzyme activity was subsequently measured with GTP the highest activity was detected, resulting from both a high Vmax and a low Km under this condition (Table III, part III).

These results suggest that relatively low GTPyS concentrations (1 μM) affect guanylate cyclase in two ways, both leading to increased cGMP synthesis in homogenates with GTP. Firstly, GTPyS lowers the Km of the enzyme for GTP. Secondly, GTPyS increases the Vmax of guanylate cyclase for GTP. At relatively high concentrations (~100 μM) GTPyS also becomes a substrate for the enzyme.

GTPyS Stimulates Particulate Guanylate Cyclase

Mn2+-dependent guanylate cyclase in Dictyostelium has been found in both soluble and particulate cell fractions (7, 9,
10, 16). To investigate which of these enzyme forms corresponded to the Mg2+-dependent enzyme and which form was activated by GTPyS, cell homogenates were made in the presence or absence of GTPyS, rapidly centrifuged, and the cyclase activity in sediment and supernatant was measured with Mg2+-GTP at pH 7.0. Measurements were also done with Mg2+ ions at pH 8.0 and with Mn2+/2 mM GTP, to investigate whether the enzyme activity observed under these conditions behaved differently from the activity detected with Mg2+-GTP at pH 7.0.

Mg2+-dependent guanylate cyclase was found both in pellet and supernatant fractions after 1 min centrifugation at 10,000 × g (Fig. 5). When homogenates prepared in the presence of GTPyS were fractionated, increased guanylate cyclase activity was measured only in 10,000 × g pellets, not in supernatants (Fig. 5). The same results were obtained when the enzyme was measured with Mg2+ ions at pH 8.0 and with Mn2+/2 mM GTP. No evidence was found, using these assay conditions, for a guanylate cyclase other than the Mg2+-
dependent enzyme and behaving differently from the latter enzyme. Only, measuring at pH 8.0 or with Mn2+/2 mM GTP, the activity in preparations not containing GTPyS was higher than when measured with Mg2+-GTP at pH 7.0 (Fig. 5). This apparently results from a higher intrinsic activity of Mg2+-dependent guanylate cyclase.

**DISCUSSION**

In this paper we report on some of the characteristics of a highly active Mg2+-dependent guanylate cyclase in *D. discoideum* membranes. This enzyme may be identical to the enzyme regulated in *vivo* via cell surface receptors, as we suggested before (11). The enzyme is stimulated in *vivo* by the GTP analogues GTPyS and Gpp(NH)p, the ATP analogue App(NH)p, and inhibited by nanomolar concentrations of Ca2+ ions. These effects may reflect regulatory mechanisms that operate in *vivo*, especially because they were established under *in vitro* conditions that are more physiologic than were used previously, i.e. using Mg2+-GTP instead of Mn2+-GTP as a substrate for the enzyme. Optimal enzyme activity was obtained in homogenates, immediately after lysis of cells in the presence of GTPyS, App(NH)p, and the Ca2+ chelator EGTA. Under these conditions a *V*max = 66 pmol/min/107 cells was estimated with Mg2+-GTP as substrate. This activity is close to the activity estimated for guanylate cyclase in *vivo* that is stimulated via cell surface receptors (60–90 pmol/min/107 cells; 12, 19, 23).

Several mechanisms can be envisaged to explain the higher activity of Mg2+-dependent guanylate cyclase in the presence of GTPyS: 1) GTPyS may be a substrate for the enzyme; 2) GTPyS may be stabilizing the enzyme; and 3) GTPyS may be an allosteric activator. There is evidence for each of these mechanisms.

(a) That GTPyS might be a substrate is strongly suggested by the observation of GTPyS-dependent cGMP synthesis, characterized by a *V*max similar to the *V*max with GTP (Table III). This GTPyS-dependent synthesis was obtained when GTPyS was the sole (exogenous) guanosine triphosphate in the reaction.

(b) In favor of the idea that GTPyS stabilizes the enzyme is the observation that the presence of GTPyS during cell lysis resulted in higher cGMP synthesis than presentation of GTPyS only at the time of the reaction (Fig. 2). Furthermore, the enzyme activity decayed more rapidly in the absence than in the presence of GTPyS (Fig. 1).

(c) Suggesting allosteric activation of guanylate cyclase by GTPyS, finally, is the observation that in the presence of GTPyS the enzyme has a significantly lower *Km* for substrate than in its absence (Table III). This lower *Km* was observed when GTPyS was used as the sole substrate for the enzyme, as well as with GTP as a substrate in the presence of a subsaturating concentration of GTPyS (Table III, part III). The kinetic data rule out the possibility that the higher activity of the enzyme under the latter conditions was only because GTPyS was a substrate in addition to GTP.

We propose the following explanation for the effect of GTPyS on the activity of Mg2+-dependent guanylate cyclase in homogenates. In addition to the catalytic site there is an allosteric site on the enzyme with high affinity for guanine nucleotides. This allosteric site may be located on the enzyme itself or on a separate component, e.g. a G-protein (24). Occupation of the allosteric site by GTPyS lowers the *Km* of the enzyme and in addition leads to its stabilization. The latter effect is observed as an increase in the *V*max after some time of incubation with GTPyS, for instance of the labile enzyme that is present in homogenates immediately after cell lysis. GTPyS is also a substrate of the enzyme and therefore does not competitively inhibit the cGMP synthesis when GTP is present as substrate. GTP apparently has a much lower affinity for the allosteric site than GTPyS. The affinity of the enzyme for the natural substrate GTP is increased by the presence of GTPyS because this nucleotide occupies the allosteric site more permanently than GTP. Whether this occurs by a mechanism analogous to G-proteins (24) remains to be investigated. As yet, we have no direct evidence for the involvement of G-proteins in the regulation of Mg2+-
dependent guanylate cyclase; the properties of the enzyme were not found to be altered by treatment of cells with cholera or pertussis toxin (data not shown).

The role of Ca2+ in the regulation of guanylate cyclase appears to be contrary to the role of guanine nucleotides, i.e. inhibitory. It appears that the Mg2+-dependent guanylate cyclase is much more sensitive to Ca2+ than the Mn2+-dependent enzyme, as Mn2+-dependent guanylate cyclase is only weakly inhibited by Ca2+ ions (9). Micromolar concentrations of Ca2+ ions seem to be released in the cytoplasm of *D. discoideum* cells in response to receptor-induced activation of a phosphatidylinositol cycle (21). The released micromolar concentrations of Ca2+ ions would be sufficient for a complete arrest of the enzyme, as is suggested by the sensitivity of the Mg2+-dependent enzyme for Ca2+ ions. Thus, a basal Ca2+ concentration in the cytoplasm of 100 nM or more might keep guanylate cyclase in an inactive state. An interesting possibility is that receptor-mediated stimulation of guanylate cyclase in *vivo* is brought about by a temporal relaxation of a tonic inhibition of the enzyme by Ca2+ ions.

The effects of Ca2+ ions and Ins(1,4,5)P3 we observed on cGMP synthesis in *vivo* are opposite to those reported by Europe-Finner et al. (3, 4) with permeabilized *D. discoideum* cells. In contrast to these authors, we only observed inhibitory effects of Ca2+ ions on guanylate cyclase. We were unable to reproduce their results with Ca2+ ions in permeabilized cells, while we found only very weak responses with Ins(1,4,5)P3. We have at present no explanation for the apparently differing findings. The present results are a first characterization of a guanylate cyclase from *D. discoideum* that can be assayed in *vivo* with a substrate, probably identical to the physiological substrate, Mg2+-GTP. They should provide a basis for further in *vivo* studies on the regulation of the enzyme.

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REFERENCES
1. Janssens, P. M. W., and Van Haastert, P. J. M. (1987) Microbiol. Rev. 51, 396–418
2. Janssens, P. M. W. (1987) Trends Biochem. Sci. 12, 456–459
3. Europe-Finner, G. N., and Newell, P. C. (1985) Biochem. Biophys. Res. Commun. 130, 1115–1122
4. Small, N. V., Europe-Finner, G. N., and Newell, P. C. (1986) FEBS Lett. 203, 11–14
5. Van Haastert, P. J. M., Kesbeke, F., Reymond, C. D., Firtel, R. A., Luderm, E., and Van Driel, R. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 4905–4909
6. Janssens, P. M. W. (1988) Comp. Biochem. Physiol. 90A, 209–223
7. Mato, J. M., and Malchow, D. (1978) FEBS Lett. 90, 119–122
8. Mato, J. M. (1979) Biochem. Biophys. Res. Commun. 88, 569–574
9. Padh, H., and Brenner, M. (1984) Arch. Biochem. Biophys. 229, 73–80
10. Ward, A., and Brenner, M. (1977) Life Sci. 21, 997–1008
11. Janssens, P. M. W., and de Jong, C. C. C. (1988) Biochem. Biophys. Res. Commun. 150, 405–411
12. Van Haastert, P. J. M., and Van der Heijden, P. R. (1983) J. Cell Biol. 96, 547–553
13. Theibert, A., and Devreotes, P. N. (1986) J. Biol. Chem. 261, 15121–15125
14. Padh, H., and Brenner, M. (1985) J. Biol. Chem. 260, 3613–3616
15. Otte, A. P., Plomp, M. J. E., Arents, J. C., Janssens, P. M. W., and Van Driel, R. (1986) Differentiation 32, 185–191
16. Janssens, P. M. W., Van Essen, H. W., Guijt, J. J. M., De Waal, A., and Van Driel, R. (1987) Mol. Cell. Biochem. 76, 55–65
17. Tovey, K. C., Oldham, K. G., and Whelan, J. A. M. (1974) Clin. Chim. Acta 56, 221–234
18. Coukell, M. B., Cameron, A. M., Pitre, C. M., and Mee, J. D. (1984) Den. Biol. 103, 246–257
19. Van Haastert, P. J. M., Van Lookeren Campagne, M. M., and Ross, F. M. (1982) FEBS Lett. 147, 149–152
20. Bartfai, T. (1979) Adv. Cyclic Nucleotide Res. 10, 219–242
21. Europe-Finner, G. N., and Newell, P. C. (1986) Biochim. Biophys. Acta 887, 335–340
22. Van Haastert, P. J. M., Snaar-Jagalska, B. E., and Janssens, P. M. W. (1987) Eur. J. Biochem. 162, 251–258
23. Ross, F. M., and Newell, P. C. (1981) J. Gen. Microbiol. 127, 339–350
24. Neer, E. J., and Clapham, D. E. (1988) Nature 333, 129–134