

Guanylate Cyclase Activity in Permeabilized *Dictyostelium discoideum* Cells

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Abstract *Dictyostelium discoideum* cells respond to chemoattractants by transient activation of guanylate cyclase. Cyclic GMP is a second messenger that transduces the chemotactic signal. We used an electropermeabilized cell system to investigate the regulation of guanylate cyclase. Enzyme activity in permeabilized cells was dependent on the presence of a nonhydrolysable GTP analogue (e.g., GTP γ S), which could not be replaced by GTP, GDP, or GMP. After the initiation of the guanylate cyclase reaction in permeabilized cells only a short burst of activity is observed, because the enzyme is inactivated with a $t_{1/2}$ of about 15 s. We show that inactivation is not due to lack of substrate, resealing of the pores in the cell membrane, product inhibition by cGMP, or intrinsic instability of the enzyme. Physiological concentrations of Ca²⁺ ions inhibited the enzyme (half-maximal effect at 0.3 μ M), whereas InsP₃ had no effect. Once inactivated, the enzyme could only be reactivated after homogenization of the permeabilized cells and removal of the soluble cell fraction. This suggests that a soluble factor is involved in an autonomous process that inactivates guanylate cyclase and is triggered only after the enzyme is activated. The initial rate of guanylate cyclase activity in permeabilized cells is similar to that in intact, chemotactically activated cells. Moreover, the rate of inactivation of the enzyme in permeabilized cells and that due to adaptation *in vivo* are about equal. This suggests that the activation and inactivation of guanylate cyclase observed in this permeabilized cell system is related to that of chemotactic activation and adaptation in intact cells. © 1996 Wiley-Liss, Inc.

Key words: *Dictyostelium*, guanylate cyclase, cGMP, chemotaxis, GTP, adaptation

The cellular slime mould *Dictyostelium discoideum* displays a life cycle in which unicellular amoebae proliferate as long as nutrients are available. A precise, genetically defined develop-

mental process is initiated by starvation. Development starts with the aggregation of cells into a multicellular organism that consists of two major, differentiated cell types: spores and stalk cells [Loomis, 1975, 1982; Schaap, 1991]. Throughout development cells communicate by exchanging extracellular cAMP signals. During starvation cAMP is secreted in a pulsatile manner. This signal not only induces a chemotactic response resulting in cell aggregation, but it also initiates and synchronizes specific developmental events.

Binding of cAMP to highly specific cell surface receptors results in activation of adenylate cyclase, among other biochemical responses. Most of the newly synthesized cAMP is secreted, resulting in amplification and relay of the chemotactic signal [Loomis, 1982; Van Haastert, 1984; for a review see Gross, 1994]. Cyclic AMP receptors are also coupled to guanylate cyclase [Keskibeke and Van Haastert 1985; Van Haastert, 1987]. Binding of cAMP to its receptor results in a very rapid, transient activation of guanylate cyclase [Mato and Malchow, 1978]. The intracellular cGMP concentration reaches its maximal

Abbreviations used: AppNHp, adenosine 5'-[β , γ ,-imido] triphosphate; cAMP, adenosine 3',5'-monophosphate; cGMP, guanosine 3',5'-monophosphate; dcAMP, 2' deoxyadenosine 3',5'-monophosphate; DTT, dithiothreitol; EGTA, ethylene glycol bis(β -amino ethyl ether)-N,N,N',N'-tetra acetic acid; GDP, guanosine 5'-diphosphate; GMP, guanosine 5'-monophosphate; GTP, guanosine 5'-triphosphate; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); Hepes, N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid; InsP₃, D-myo-inositol 1,4,5-trisphosphate.

Enzymes used: adenylate cyclase, (EC 4.6.1.1); cGMP phosphodiesterase, (EC 3.1.4.3.5); guanylate cyclase, (EC 4.6.1.2). Received May 19, 1995; accepted August 4, 1995.

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value 10 s after stimulation [Watts and Ashworth, 1970; Mato et al., 1977; Mato and Malchow, 1978; Van Haastert, 1987]. Due to rapid adaptation of the cGMP response and breakdown of cGMP by a cGMP-specific phosphodiesterase, the intracellular cGMP concentration returns to the resting level within 30 s [Mato et al., 1977]. Evidence has been presented that indicates that the cAMP-induced, transient rise in cGMP concentration is tightly linked to the chemotactic response [Ross and Newell, 1981; Van Haastert et al., 1982; Hall et al., 1989; Liu and Newell, 1988, 1991, 1994; Newell and Liu 1992].

In certain mammalian cell types, cell surface receptors and guanylate cyclase have been found integrated in a single transmembrane polypeptide [Schulz et al., 1989; for reviews see Garbers, 1994; Garbers et al., 1994]. No evidence for this type of cell surface receptor has been found in *Dictyostelium* yet [Tao et al., 1992]. So far, all identified cAMP receptors belong to the family of the G-protein linked receptors, that are typified by seven transmembrane segments [Saxe et al., 1993; Johnson et al., 1993]. Little is known about the molecular mechanisms that control guanylate cyclase activity in *D. discoideum*.

We have developed a method [Schoen et al., 1989] to permeabilize *Dictyostelium* cells, using electropermeabilization [Knight and Baker, 1982; Sugar et al., 1987]. This gentle procedure leaves cell organelles undamaged. After permeabilization, low molecular weight components have access to the cytoplasmic compartment, whereas proteins and other macromolecules are retained inside the cell.

Here we investigate the properties of guanylate cyclase in electropermeabilized cells. We find that the enzyme is inactivated in about 30 s. Inactivation of guanylate cyclase could only be reversed after homogenization of the cells and subsequent removal of a soluble cell fraction. Furthermore, guanylate cyclase activity requires the presence of a nonhydrolysable GTP analogue in addition to the substrate GTP. Guanylate cyclase is inhibited by submicromolar concentrations of Ca^{2+} . Results are discussed in the context of regulation of the enzyme in vivo.

EXPERIMENTAL PROCEDURES

Materials

Guanosine 3',5'-monophosphate (cGMP), 2'-deoxyadenosine 3',5'-monophosphate (dcAMP), GTP, GDP, GMP, $\text{GTP}\gamma\text{S}$, guanosine 5'-[β,γ ,

imido] triphosphate (GppNHp), and adenosine 5'-[β,γ ,imido] triphosphate (AppNHp) were obtained from Boehringer (Mannheim, Germany). The radiochemicals 2'-deoxy-D-[^{14}C]glucose ($11.1 \text{ TBq} \cdot \text{mol}^{-1}$), [^3H]Ins [(1,4,5) P_3 740 $\text{GBq} \cdot \text{mol}^{-1}$], and [^3H]H $_2\text{O}$ ($3.34 \text{ GBq} \cdot \text{mol}^{-1}$) were purchased from Amersham (Amersham's Hertogenbosch, The Netherlands). Polycarbonate filters with 5 μm pore size were from Nucleopore Corporation (Pleasanton, CA).

Methods

Culture conditions. *Dictyostelium discoideum* cells (axenic strain AX2) were grown in HL-5 medium, containing maltose instead of glucose [Watts and Asworth, 1970]. Cells were harvested in the late logarithmic phase at a density of approximately 5×10^6 cells/ml in 15 mM $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 6.2 (Pi-buffer), washed once, and starved in suspension in Pi-buffer at a density of 10^7 cells/ml at 23°C. After 5 h cells were washed twice in Pi-buffer, and the final pellet was resuspended in Pi-buffer to a density of 10^8 cells/ml. Cells were either used immediately or were kept on ice under continuous aeration for at most 1 h before starting the experiment.

Electropermeabilization and cell lysis.

Cell permeabilization was carried out essentially as described by Schoen et al. [1989]. Aggregation-competent cells were collected by centrifugation (3 s, 10,000g), the pellet was resuspended in 20 mM Hepes-NaOH (pH 7.5), 100 mM sucrose, 10 mM K-acetate, 100 μM AppNHp, and 0.5 mM MgSO_4 at a density of 1×10^8 cells/mL (standard electropermeabilization conditions). One milliliter of the cell suspension was pipetted into the electroporation chamber between two parallel platinum electrodes, each with a surface area of 1 cm^2 , positioned 1 cm apart. Permeabilization was achieved by exposing the cells to seven square wave pulses of 50 μs duration each (rise and fall times were approximately 50 ns) of 2 kV at 0°C under continuous stirring. After seven discharges, applied at 3 s intervals, the temperature increased only 1–2°C. The extent of permeabilization was estimated visually by ethidium bromide fluorescence. Nonpermeable cells excluded ethidium bromide and were not fluorescent, whereas permeable cells accumulated the dye. Under standard permeabilization conditions more than 98% of the cells became permeable. Under these conditions, no protein was released from the cells.

Cell lysates were prepared by forcing a suspension of untreated or permeabilized cells through a 5 μm pore-size polycarbonate filter [Das and Henderson, 1983; Van Haastert, 1987]. More than 98% of the cells were broken by this procedure as judged by phase contrast microscopy.

Measurement of cell permeability. After electropermeabilization the accessible cell volume was determined by measuring the rate of influx of radioactive deoxyglucose, which is nonpermeant and metabolically inert [Schoen et al., 1989]. The total intracellular volume was determined by equilibrating the permeabilized cells with tritiated water, which is a permeant molecule and has access to all cell compartments. After a certain time interval, the intra- and extracellular compartments were separated by centrifugation through a layer of silicon oil [Gerisch and Wick, 1975]. From the radioactivity in the pellet the total intracellular volume and the cellular volume accessible to the nonpermeant component could be calculated. Corrections were made for label adhering to the outside of the cells during centrifugation through silicon oil.

Measurement of guanylate cyclase activity. Guanylate cyclase activity was measured as described before [Janssens et al., 1987; Otte et al., 1986]. The standard incubation mixture (500 μl) contained 20 mM Hepes-NaOH (pH 7.5), 100 mM sucrose, 5 mM dithiothreitol, 10 mM K-acetate, 6.5 mM MgSO_4 , 5 mM GTP, 100 μM $\text{GTP}\gamma\text{S}$, 100 μM AppNHp, and 2 mM EGTA. In some experiments the free Ca^{2+} ion concentration was kept constant by using a 2 mM Ca^{2+} /EGTA buffer [Bartfai, 1979]. In experiments that concerned effects of InsP_3 , EGTA was omitted. Dithiothreitol abolished essentially all cyclic nucleotide phosphodiesterase activity in the assay system [Pannbacker and Bravard, 1972; Green and Newell, 1975]. The guanylate cyclase reaction was initiated by the addition of 250 μl homogenate or permeabilized cells (1×10^8 cell equivalents or cells per milliliter). The assay was carried out at 15°C. To measure cGMP levels at various time points, samples were quenched in perchloric acid (final concentration 1 N) and kept frozen until analysis. Perchloric acid extracts were neutralized to pH 7.0 by addition of a 1.5 M KHCO_3 , 3.5 M KOH neutralization mixture and centrifuged for 2 min at 10,000g. Cyclic GMP concentrations were measured by a radioimmuno assay, using a highly specific anti-cGMP rabbit serum, prepared against cGMP

covalently linked to bovine serum albumin [Steiner et al., 1972]. The affinity of this antiserum for cAMP and dcAMP was 10^3 -fold lower than for cGMP, whereas GTP, GDP, GMP, $\text{GTP}\gamma\text{S}$, and AppNHp were bound with a more than 10^6 -fold lower affinity.

RESULTS

Measurement of Guanylate Cyclase Activity in Permeabilized Cells

We have used electropermeabilization conditions that meet the following criteria [Schoen et al., 1989]. 1) The pores allow an efficient exchange of low molecular weight components between cytoplasm and extracellular medium. At the same time they are sufficiently small to confine proteins and other macromolecules to the intracellular space. In this way most protein-protein interactions will be maintained in the permeable cells. 2) The plasma membrane is selectively permeabilized. Organelles like the lysosomal compartment remain intact and are nonpermeable. 3) The electropermeabilization procedure results in a homogeneous population of permeabilized cells. 4) The pores are stable for at least several minutes. Figure 1 shows that the rate of influx of a low molecular weight, nonmetabolized, nonpermeant substance (^{14}C -deoxy-glucose) into permeabilized cells remained unchanged for at least 2 min after permeabilization. Various nucleotides that are present in reaction media, like GTP, cGMP and ATP, had no effect on the influx of deoxyglucose (data not shown).

We have measured guanylate cyclase activity using the physiological substrate Mg^{2+} -GTP rather than the commonly used nonphysiological Mn^{2+} -GTP. Under these conditions, guanylate cyclase activity is only found in the particulate cell fraction [Schulkes et al., 1991]. If Mn^{2+} -GTP is used, a soluble guanylate cyclase activity is also found [Mato and Malchow, 1978; Janssens et al., 1987; Padh and Brenner, 1984]. The assay conditions for the measurement of the guanylate cyclase activity were similar to those of [Janssens and De Jong, 1988; Janssens et al., 1989], but the concentration of the substrate (GTP) in the medium was 5 mM instead of 0.3 mM. The rationale for this was that intracellular GTP-ases continuously hydrolyse GTP, making the steady-state GTP level inside the permeabilized cell lower than the concentration in the extracellular fluid [Trahey and McCormick, 1987]. Extracellular GTP concentrations above

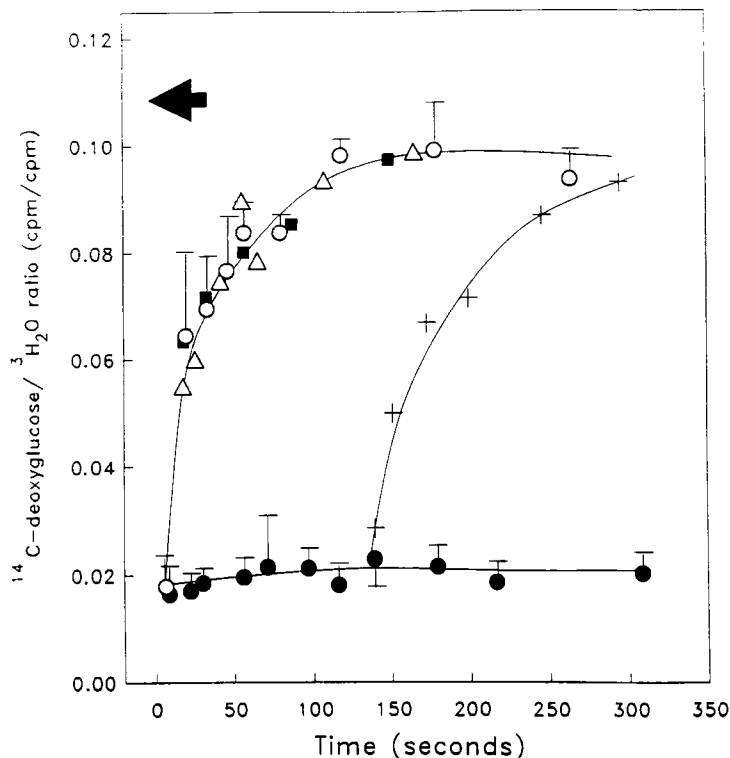


Fig. 1. Cell permeability during cGMP synthesis. The permeability of electropermeabilized cells for low molecular weight substances was determined according to Schoen et al. [1989]. Permeabilized (Δ , \blacksquare , \circ , $+$) and nonpermeabilized (\bullet) cells were incubated with the nonpermeant, nonmetabolizable substance [^{14}C]-deoxyglucose (2 mM , $2 \times 10^4\text{ cpm} \cdot \text{ml}^{-1}$) and the permeant [^3H] H_2O ($2 \times 10^5\text{ cpm} \cdot \text{ml}^{-1}$). Intracellular label was determined after centrifugation of cells through silicone oil. Appropriate corrections were made for adhering, extracellular label. The ratio of [^{14}C] over [^3H] is a measure for the rate of influx of [^{14}C]-deoxyglucose and therefore of the permeability of the cells. The filled arrow marks the ratio of [^{14}C]-deoxyglu-

cose/[^3H] H_2O in the extracellular medium, representing the theoretical maximum that would be reached if [^{14}C]-deoxyglucose had access to all intracellular compartments that are accessible to [^3H] H_2O . The rate of influx of [^{14}C]-deoxyglucose is shown immediately after electropermeabilization (Δ , \blacksquare , \circ) and 2 min after permeabilization ($+$) at $t = 0$. The effect of various nucleotides on cell permeability was investigated: standard guanylate cyclase assay conditions with no additions (\circ , \bullet), with addition of $0.20\ \mu\text{M}$ cGMP (Δ), with addition of 2 mM ATP (\blacksquare), and with 5 mM ATP instead of GTP ($+$). Data of \circ and \bullet are the mean \pm s.d. of four independent experiments.

5 mM did not increase the measured enzyme activity. In all experiments $100\ \mu\text{M}$ AppNHP was added as an activator of guanylate cyclase [Janssens and De Jong, 1988; Janssens et al., 1989] and an inhibitor of nonspecific nucleotide triphosphatases [Yount et al., 1971; Snaar-Jagalska, 1980]. An analysis of the pH dependence of the guanylate cyclase activity in permeable cells showed that the activity dramatically decreased below pH 7.0 (data not shown). Therefore, all guanylate cyclase measurements were carried out at pH 7.5.

cGMP Synthesis in Permeabilized Cells

Figure 2 shows the time course of cGMP synthesis *in vivo* and in permeabilized cells. The reaction was initiated by the addition of substrate (GTP) immediately after permeabilization.

The same result was obtained if the reaction was started 2 min after electropermeabilization. The initial synthesis rate immediately after permeabilization was about $500\text{ pmol cGMP} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. This is similar to the initial rate of cGMP production observed *in vivo* after chemotactic stimulation and in a cell lysate (Fig. 2). In permeabilized cells the rate of cGMP synthesis rapidly dropped to basal levels within 30 s. Because cGMP phosphodiesterase activity is inhibited under our assay conditions, due to the presence of DTT, the cGMP level remains essentially constant after 30 s. Evidently, guanylate cyclase in permeabilized cells is active for only a short period of time. This behaviour of the guanylate cyclase system in permeable cells is similar to that observed *in vivo*. After a chemotactic stimulation, guanylate cyclase is activated rapidly and

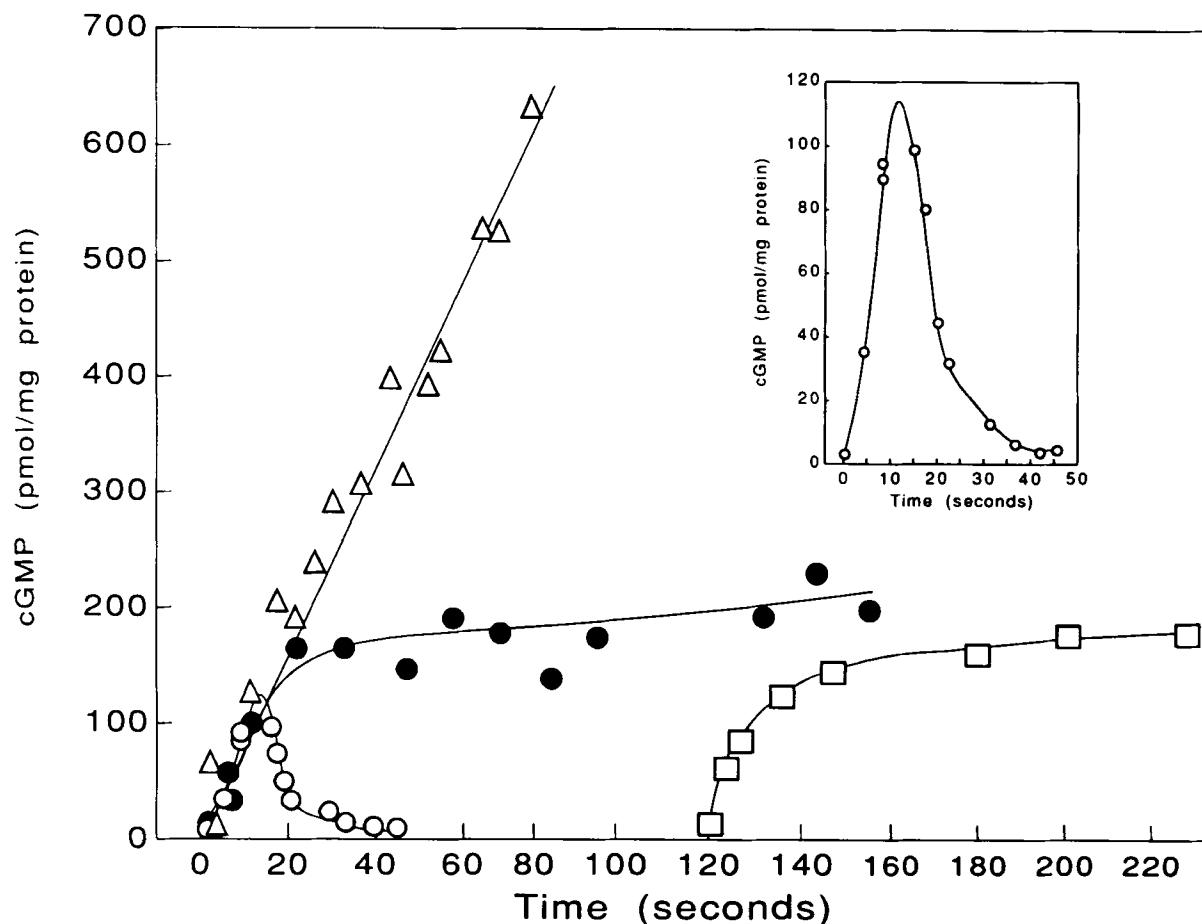


Fig. 2. Kinetics of cGMP synthesis. A suspension of 10^8 cells/ml was permeabilized or lysed under standard conditions in the presence of 0.2 mM GTP γ S. Cyclic GMP synthesis was started either immediately (\bullet) or 2 min after permeabilization (\square) by addition of reaction mixture. Cyclic GMP levels as a function of time were recorded in electroporabilized cells (\bullet), cell lysates (Δ), and intact cells (\circ). In permeabilized cells and in

the cell lysate the phosphodiesterase activity was completely inhibited by dithiothreitol (5 mM). In the permeable cells and the cell lysate the reaction was started by the addition of substrate (Mg^{2+} -GTP) at $t = 0$; the intact cells were stimulated at $t = 0$ by addition of 10 μ M dcAMP. The inset shows the in vivo response in more detail.

is subsequently switched off within 30 s, due to adaptation of the system [Van Haastert, 1987]. Because in vivo newly synthesized cGMP is rapidly degraded, a peak of cGMP is produced after cell stimulation (Fig. 2).

One conceivable mechanism for the inactivation of guanylate cyclase in permeabilized cells is instability of the guanylate cyclase enzyme. Figure 2 shows that cGMP synthesis was found to be the same for reactions that were initiated immediately after permeabilization or 2 min later. This shows that the guanylate cyclase activity and the inactivation mechanism are stable in permeabilized cells for at least 2 min. This result also shows that the pores remain open for at least 2 min. Another cause of guanylate cyclase inactivation could be product inhibi-

tion by cGMP. To test this possibility we have started the guanylate cyclase reaction in permeabilized cells in the presence of cGMP (0.2 μ M). The presence of the reaction product had no significant effect on the guanylate cyclase reaction (data not shown). Evidently, guanylate cyclase is not subject to product inhibition. Another potential cause of a decrease of the guanylate cyclase activity is lack of substrate. This cause, however, could be excluded. Addition of extra Mg^{2+} -GTP (5 mM) at 2 min after initiation of cGMP synthesis had no effect on cGMP production (data not shown).

We conclude that in permeable cells a regulatory mechanism is operative that suppresses guanylate cyclase activity within 30 s after the guanylate cyclase reaction has been started by

addition of substrate. Such inactivation process has never been observed in cell homogenates. This inactivation process in permeabilized cells has kinetic properties that are similar to the adaptation process *in vivo*.

GTP γ S is Required for Activation of Guanylate Cyclase But Not for Its Inactivation

The nonhydrolysable GTP analogue GTP γ S stimulated guanylate cyclase activity in permeabilized cells about tenfold (Fig. 3). The half-maximal effect was obtained at 70 μ M nucleotide. To examine whether GTP γ S also plays a role in guanylate cyclase inactivation, the relationship between GTP γ S concentration and the rate of guanylate cyclase inactivation was analyzed. As a measure for the rate of inactivation we used the time that was required to reduce the initial rate of cGMP synthesis to 50%. Figure 3 shows that GTP γ S had no effect on the inactivation process. Regardless of the GTP γ S concentra-

tion, and therefore also independent of the absolute rate of cGMP synthesis, the guanylate cyclase activity decreased 50% in about 15 s. These results show that the nonhydrolysable GTP analogue is required for guanylate cyclase activity in permeabilized cells but not for guanylate cyclase inactivation.

Timing and Specificity of the Stimulatory Effect of GTP γ S on Guanylate Cyclase in Permeabilized Cells

We have analyzed whether the timing of exposure of permeable cells to GTP γ S is important. Results are summarized in Table I. If cells were permeabilized in the absence of GTP γ S, the stimulatory effect of this nucleotide remained unaltered up to 2 min after permeabilization (*data not shown*). This was true, provided that the substrate of guanylate cyclase (Mg²⁺-GTP) was added after addition of GTP γ S. Another nonhydrolysable analogue, 5'-guanylyl imidotri-

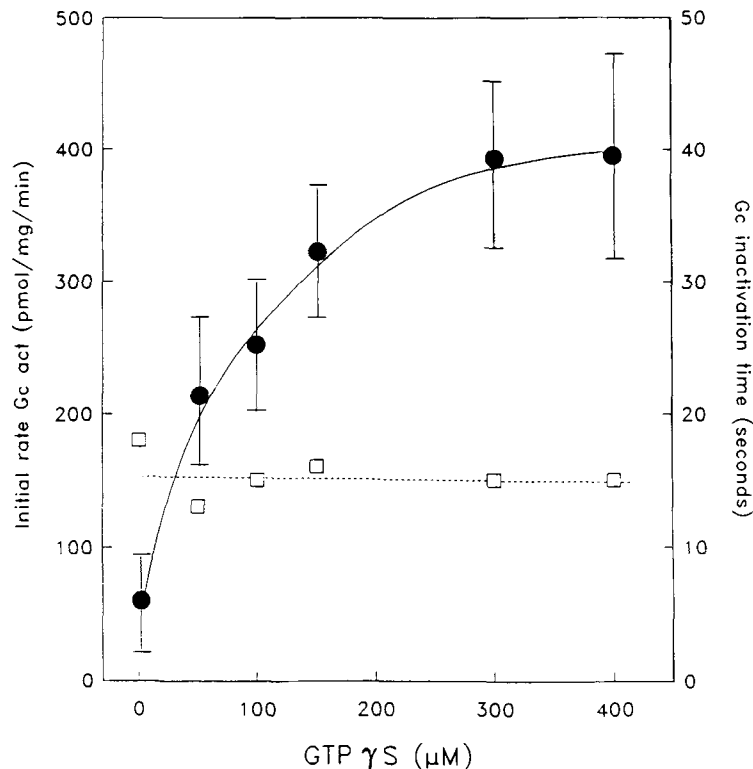


Fig. 3. Effect of GTP γ S on activation and inactivation of guanylate cyclase in electropermeabilized cells. A suspension of 10^8 cells/ml was permeabilized under standard conditions in the presence of different concentrations of GTP γ S. The same concentrations of GTP γ S were maintained during the guanylate cyclase reaction. The initial rate of cGMP synthesis is plotted vs. the GTP γ S concentration (●). Data represent the mean \pm s.d.

of four independent experiments. Immediately after the onset of the guanylate cyclase reaction, the reaction rate started to decrease due to inactivation of guanylate cyclase. As a measure for the rate of guanylate cyclase inactivation we used the time that was required for a 50% decrease of the guanylate cyclase reaction rate. The rate of inactivation (□) is independent of the GTP γ S concentration.

phosphate, had a similar effect as GTP γ S [Janssens and De Jong, 1988] (data not shown). If GTP (5 mM) was added together with or before GTP γ S (100 μ M), the stimulatory effect of the nonhydrolysable analogue was almost completely abolished (Table I). In contrast, GMP (1 mM) and GDP (1 mM) did not affect the GTP γ S stimulation of guanylate cyclase. Neither the initial rate of guanylate cyclase activity nor the rate of guanylate cyclase inactivation was changed by GDP and GMP (data not shown). Importantly, GTP (5 mM) could not replace GTP γ S (100 μ M) in the activation of guanylate cyclase (Table I).

We tested whether GTP γ S stimulates guanylate cyclase activity because it is a more efficient substrate than GTP. This is not the case. If Mg $^{2+}$ -GTP is omitted from the reaction mixture,

the enzyme activity was 4.5 times lower than in the presence of the natural substrate (Table I). It may be that GTP γ S activates a cGMP phosphodiesterase activity. This was tested by replacing Mg $^{2+}$ -GTP in the standard guanylate cyclase assay by cGMP (0.2 μ M). Subsequently we measured the residual rate of degradation of cGMP, which was only 8.5 ± 2 pmol \cdot min $^{-1}$ \cdot mg protein $^{-1}$ (n = 6) under the prevailing conditions. This value was the same in the absence and in the presence of 100 μ M GTP γ S. If the phosphodiesterase inhibitor (dithiothreitol) was omitted, the rate of cGMP degradation was 54 ± 3 pmol \cdot min $^{-1}$ \cdot mg protein $^{-1}$ (n = 6). Finally, it is possible that we measure guanylate cyclase activity in a residual population of intact, nonpermeabilized cells. We showed that cGMP synthesis in intact cells is not stimulated by GTP γ S (100 μ M) (Table I) or Mg $^{2+}$ -GTP (5 mM) (data not shown). Since cGMP synthesis in our suspensions of permeabilized cells largely depends on the presence of these nucleotides, it is unlikely that a residual fraction of intact cells contributes significantly to the guanylate cyclase activity that we measure here. These results show that nonhydrolyzable GTP analogues, like GTP γ S, activate guanylate cyclase in permeabilized cells. This nucleotide cannot be replaced by GTP, GDP, or GMP.

TABLE I. Specificity of GTP γ S Effect on Guanylate Cyclase Activity

Guanine nucleotides present during electropermeabilization	Guanine nucleotides added to the guanylate cyclase reaction mixture in addition to 5 mM Mg $^{2+}$ -GTP	Initial rate of cGMP synthesis (pmol/mg protein min)
GTP γ S (0.1 mM)	GTP γ S (0.1 mM)	504 \pm 4
—	GTP γ S (0.1 mM)	46 \pm 18
—	—	20 \pm 5
GTP (5 mM)	—	54 \pm 9
GTP γ S (0.1 mM) + GTP (5 mM)	GTP γ S (0.1 mM)	74 \pm 24
GTP γ S (0.1 mM) + GDP (1 mM)	GTP γ S (0.1 mM) + GDP (1 mM)	417 \pm 89
GTP γ S (0.1 mM) + GMP (1 mM)	GTP γ S (0.1 mM) + GMP (1 mM)	532 \pm 41
GTP γ S (0.1 mM)	GTP γ S (0.1 mM) (no substrate Mg $^{2+}$ -GTP added)	112 \pm 15
No electropermeabilization	GTP γ S (0.1 mM)	< 10

A suspension of 10^8 cells/ml was permeabilized under standard conditions as described in Methods in the presence of components given in the first column. Cyclic GMP synthesis was started immediately after electropermeabilization by addition of the standard assay mixture with additions indicated in the second column. All concentrations given are final concentrations at that particular stage of the experiment. The data in the third column are the mean \pm S.D. of three independent experiments carried out in duplicate.

Effects of InsP $_3$ and Ca $^{2+}$ Ions on Guanylate Cyclase Activity in Permeabilized Cells

Figure 4 shows that, as expected, electropermeabilized cells rapidly equilibrated with exogenous InsP $_3$. InsP $_3$ is stable for more than 10 min in permeabilized cells. InsP $_3$ (10 μ M) had no effect on the kinetics of cGMP production (Fig. 5).

Figure 6 shows that free Ca $^{2+}$ ions are a potent inhibitor of guanylate cyclase activity in electropermeabilized cells. Half-maximal inhibition was obtained in the physiological concentration range (i.e., at about 0.3 μ M free Ca $^{2+}$). Calcium ions only affected the initial rate of cGMP synthesis but not the rate of inactivation of guanylate cyclase (Fig. 6). These results suggest that Ca $^{2+}$ is a regulator of *Dictyostelium* guanylate cyclase activity, whereas InsP $_3$ is not.

Inactivation of Guanylate Cyclase in Permeabilized Cells Is Reversible

In the previous sections we have shown that in permeabilized cells guanylate cyclase is inactivated by a process that is triggered only after

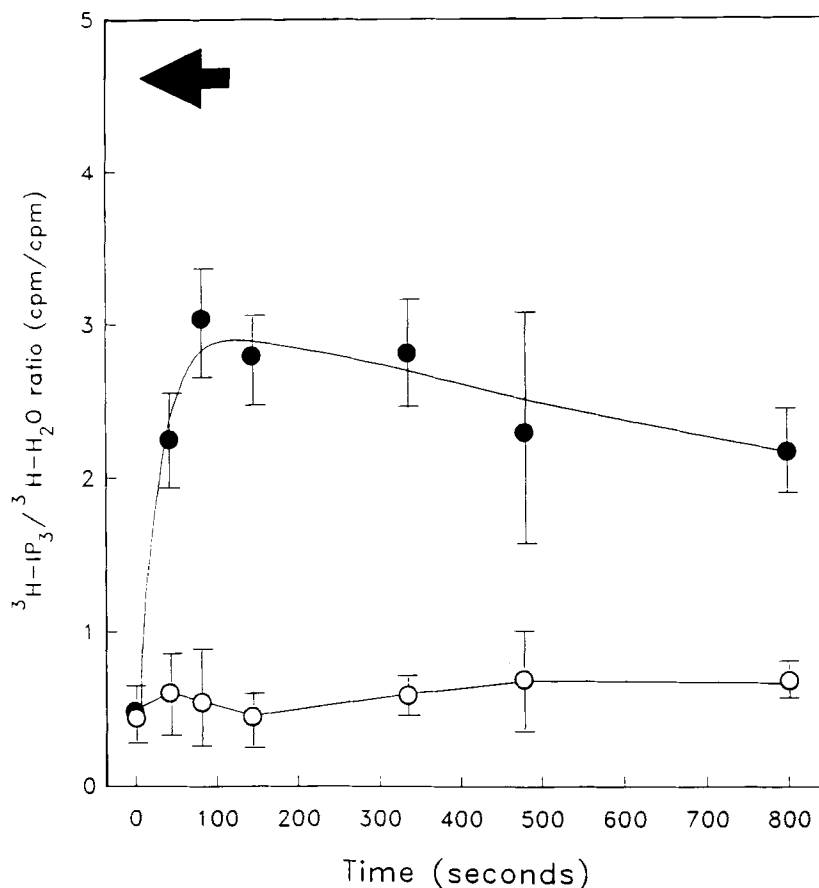


Fig. 4. Influx of InsP_3 into electropermeabilized cells. A suspension of 10^8 cells/ml in standard electropermeabilization buffer was split in two equal parts. $^3\text{H}\text{H}_2\text{O}$ (final concentration 7.7×10^5 cpm/ml) was added to one part and $^3\text{H}\text{InsP}_3$ (final concentration $10 \mu\text{M}$, 3.5×10^6 cpm/ml) to the other half. The cells were electropermeabilized under standard conditions. Intracellular, labeled H_2O and InsP_3 were determined after centrifugation through silicone oil. The ratio of intracellular labeled water (which has access to all cellular compartments) and labeled InsP_3 (entering the cell exclusively through the

pores in the plasma membrane created by electropermeabilization) has been plotted as a function of time after electropermeabilization (●). Open circles (○) represent the ratio of $^3\text{H}\text{InsP}_3 / ^3\text{H}\text{H}_2\text{O}$ of the control experiment carried out after omitting the electropermeabilization step. The filled arrow indicates the ratio of ($^3\text{H}\text{InsP}_3$ to $^3\text{H}\text{H}_2\text{O}$) in the extracellular medium, representing the theoretical maximum that would be reached if $^3\text{H}\text{InsP}_3$ had access to all intracellular compartments that are accessible to $^3\text{H}\text{H}_2\text{O}$. The experiment was carried out in triplicate (\pm s.d.).

activation of the enzyme. After inactivation, the enzyme cannot be reactivated by homogenization of the permeabilized cells (Fig. 7a). However, if the soluble fraction is removed after centrifugation, a considerable fraction of the guanylate cyclase activity can be recovered: 56% ($\pm 8\%$, S.D; $n = 7$) of the activity in a cell homogenate (Fig. 7b, bars C and D). We conclude that removal of a soluble factor is sufficient to reactivate the enzyme at least partially.

DISCUSSION

Cyclic GMP is an important second messenger in the transduction of chemotactic signals in *D. discoideum* cells [Newell and Liu, 1992]. Chemotactic receptors for cAMP and for folic

acid are functionally linked to guanylate cyclase. This enzyme is activated within seconds after chemotactic stimulation. Subsequently, cGMP synthesis is stopped after 15 s due to adaptation. The molecular mechanisms of activation of guanylate cyclase and of the adaptation process are unknown. Here we investigate the properties of the guanylate cyclase system in electropermeabilized cells. In this permeabilized cell system, small molecules like nucleotides can efficiently equilibrate between the extracellular compartment and the cytoplasmic compartments [Schoen et al., 1989, 1992]. In contrast, macromolecules like proteins are retained inside the cell. Many protein-protein interactions will therefore remain intact.

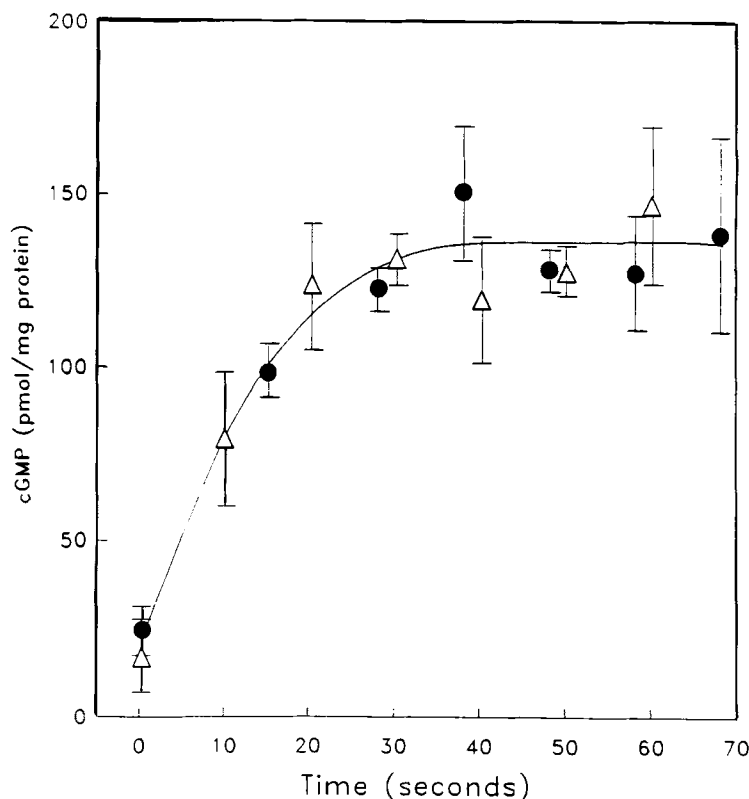


Fig. 5. Absence of an effect of InsP_3 on guanylate cyclase activity. A suspension of 10^8 cells/ml was permeabilized under standard conditions in the presence of 0.1 mM $\text{GTP}\gamma\text{S}$. This experiment was performed in the absence of EGTA and of exogenous Ca^{2+} . Cyclic GMP synthesis was started immediately after electroporation by addition of the reaction mixture in the presence (Δ) or absence (\bullet) of 10 μM InsP_3 . Data are the means of determinations in duplicate, reproduced twice.

Several properties of guanylate cyclase in permeabilized cells are similar to those observed in vivo. First of all, the initial rate of cGMP synthesis after starting the reaction by the addition of substrate (GTP) is similar to that observed in vivo after chemotactic stimulation. Subsequently, the rate of cGMP production rapidly declines. After 30 s the reaction is halted completely. Trivial explanations for these phenomena, like lack of substrate, closure of the pores in the cell membrane, product inhibition, and intrinsic instability of the enzyme, could be ruled out. Analysis of the inactivation process showed that 1) it is triggered only after the enzyme has been activated, 2) the $t_{1/2}$ of inactivation is about 15 s, and 3) the rate of inactivation is independent of the absolute rate of cGMP synthesis and of the concentration of various activators and inhibitors, like $\text{GTP}\gamma\text{S}$ or Ca^{2+} . Such autonomous inactivation process has never been observed in cell homogenates [Janssens and De Jong, 1988; Janssens et al., 1989]. Evidently, guanylate cyclase in permeable cells is capable of

a single burst of cGMP during about 30 s after the reaction is initiated by the addition of substrate. We have not found conditions to activate the enzyme in permeabilized cells a second time. Partial reactivation of the enzyme was possible only after homogenization and subsequent removal of the soluble cell fraction. This suggests that a soluble factor is involved in the inactivation of guanylate cyclase activity. Evidently, this factor is not effective in a cell homogenate, most probably because its concentration in the homogenate is too low.

The short burst of cGMP synthesis in permeabilized cells is similar to that observed after chemotactic stimulation of intact cells. In cell homogenates the guanylate cyclase behaves quite differently, producing cGMP at a constant rate for at least several minutes. The initial rates of cGMP synthesis are approximately the same in permeabilized cells and in intact cells (about 500 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$). Also the rate of inactivation in permeabilized cells and that of adaptation in intact cells are about equal ($t_{1/2}$ is

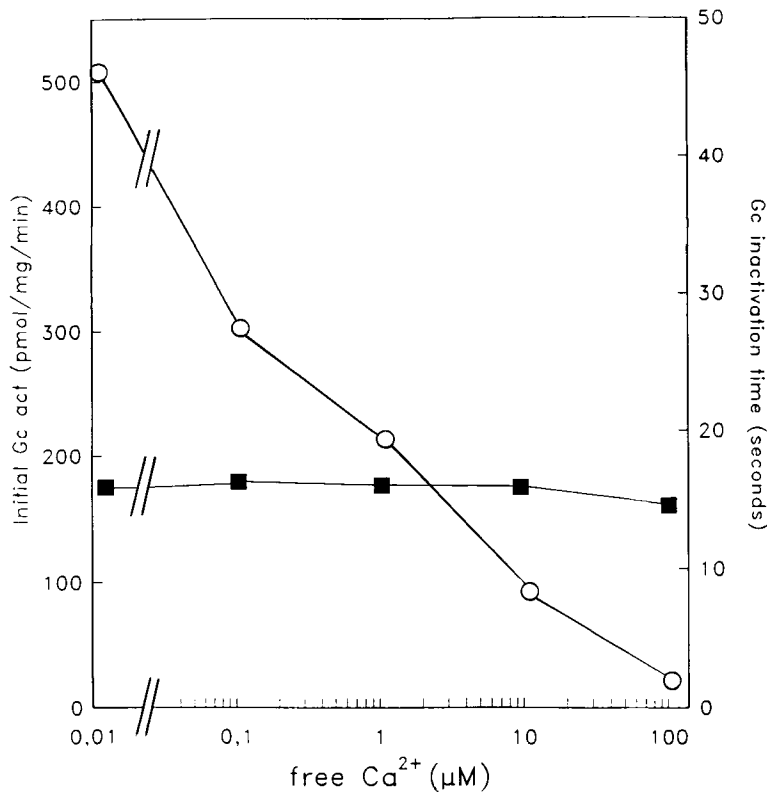


Fig. 6. Effect of Ca^{2+} ions on guanylate cyclase activity in electropermeabilized cells. A suspension of 10^8 cells/ml was electropermeabilized under standard conditions. After permeabilization, cells were resuspended in the reaction mixture in which the free Ca^{2+} concentration was controlled by a 2 mM Ca^{2+} /EGTA buffer. The initial guanylate cyclase activities (○) at different free Ca^{2+} concentrations are shown. The rate of guanylate cyclase inactivation (■) was determined as described for Fig. 3.

about 15 s). Therefore, it is tempting to speculate that the inactivation process in electropermeabilized cells is related to adaptation. If true, we may conclude that 1) the adaptation process is triggered only if the enzyme is active, 2) the intracellular messengers Ca^{2+} and InsP_3 , the activator of guanylate cyclase $\text{GTP}\gamma\text{S}$, and the absolute rate of cGMP synthesis do not affect the kinetics of the adaptation process, and 3) adaptation is mediated by a soluble factor that after removal allows the system to become active again. This latter observation may be related to the deadaptation process. The permeabilized cell system that we describe here creates an excellent opportunity to investigate adaptation of the chemotactic cGMP response in molecular detail.

Guanylate cyclase activity in permeabilized cells had an almost absolute requirement for the nonhydrolysable GTP analogue $\text{GTP}\gamma\text{S}$, which is very similar to what is observed cell lysates [Schulkes et al., 1992]. Another GTP analogue, GppNHp , had the same effect (not shown). GTP

could not replace $\text{GTP}\gamma\text{S}$, even if present at a fiftyfold higher concentration than $\text{GTP}\gamma\text{S}$. If added before or at the same time as $\text{GTP}\gamma\text{S}$, GTP abolished the stimulatory effect of the nonhydrolysable analogue. Since the K_m of guanylate cyclase for GTP and $\text{GTP}\gamma\text{S}$ differs only by a factor of three [Janssens et al., 1989], the nonhydrolysable analogue evidently acts via a guanine nucleotide binding site that is different from the catalytic site of the enzyme. A likely interpretation is that GTP itself is not able to activate guanylate cyclase via the allosteric receptor site, because the nucleotide is rapidly hydrolysed and the product (GDP) does not exchange readily with free triphosphates. $\text{GTP}\gamma\text{S}$, in contrast, cannot be hydrolysed and is therefore able to activate the GTP receptor permanently. $\text{GTP}\gamma\text{S}$ is stable in the time course of our experiments [F. Eckstein, personal communication]. The properties described here are typical for G-protein-like regulators. An interesting observation in this respect was made by Martin et al. [1986], who found that the generation of inosi-

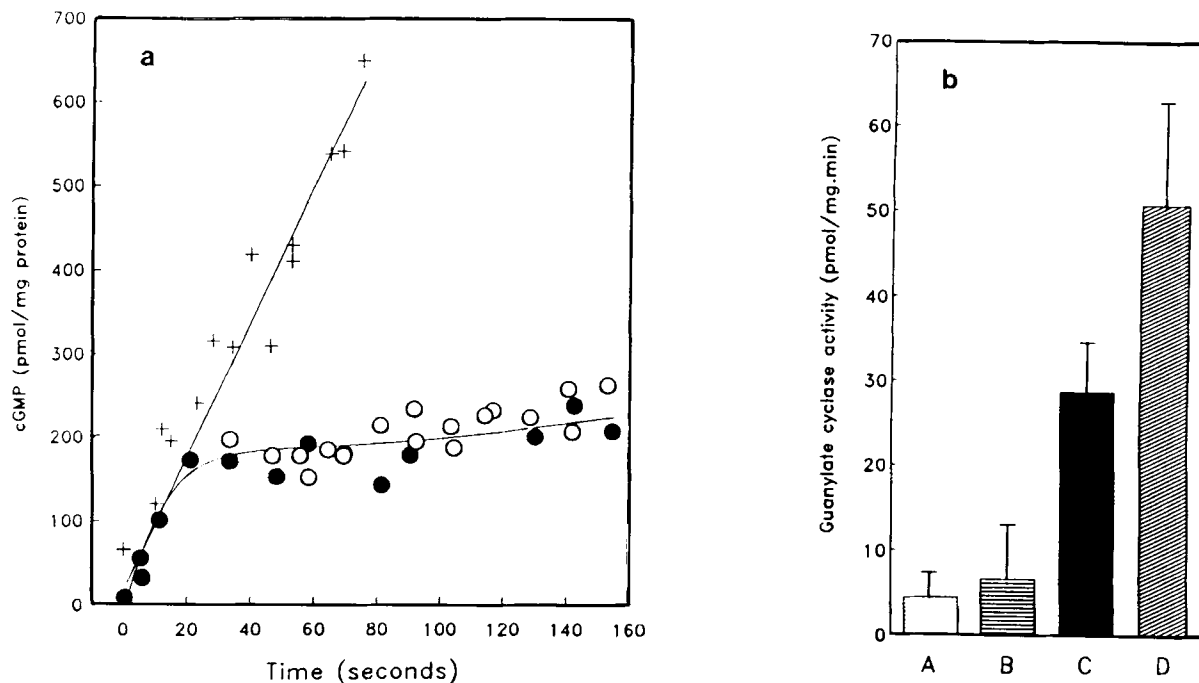


Fig. 7. Partial reactivation of inactivated guanylate cyclase. **a:** Time course of cGMP synthesis. ●: Permeabilized cells in which the guanylate cyclase reaction was started at $t = 0$, immediately after permeabilization, and the GMP production was measured for 2.5 min. ○: Permeabilized cells in which the guanylate cyclase reaction was started at $t = 0$ were lysed at $t = 30$ s, after which the cGMP production was measured over a period of 2 min. +: Cell lysate in which the guanylate cyclase reaction was started immediately after lysis of intact cells. **b:** Rate of the guanylate cyclase reaction. *Bar A:* Cells were permeabilized and the guanylate cyclase reaction was started at $t = 0$, immediately after permeabilization (same conditions as ● in panel a). The bar represents the rate of cGMP synthesis at $t = 5$ min. *Bar B:* Cells were permeabilized and the guanylate cyclase reaction was started at $t = 0$. At $t = 5$ min the cells were lysed,

and the particulate fraction was centrifuged for 1 min at 10,000g at room temperature and resuspended in its own supernatant. Immediately after this procedure the rate of cGMP production was determined. *Bar C:* Same conditions as in B, except that after the centrifugation step the supernatant was removed and the particulate fraction was resuspended in buffer of the same ionic composition and nucleotide concentration as the supernatant in B. Subsequently, the rate of cGMP production was measured immediately. *Bar D:* Activity in particulate fraction of cell lysate made at $t = 0$ and measured at $t = 5$ min. Buffer conditions in lysates and in suspensions of permeabilized cells were identical. Reaction conditions for determining the rates of cGMP production were as described in Methods section. All experiments were carried out seven times in duplicate (\pm s.d.).

tol phosphate in an electropermeabilized cell system could be potentiated by micromolar concentrations of $GTP\gamma S$. In contrast, addition of GTP even in the millimolar range was ineffective. Interestingly, the Rho family of GTP binding proteins has been shown to translocate to the cell membrane in the $GTP\gamma S$ -bound state and activate the membrane-associated NADPH oxidase in neutrophils [Bokoch et al., 1994]. It is conceivable that similar small G-proteins are involved in *D. discoideum* cell signal transduction via guanylate cyclase. Whatever the molecular mechanism is, our results show clearly that guanosine triphosphate is not only a substrate but also a regulator of guanylate cyclase activity.

Our data indicate that Ca^{2+} is a potent inhibitor of guanylate cyclase (half-maximal inhibi-

tion at $0.3 \mu M$). Janssens et al. [1989] showed a similar inhibitory effect of Ca^{2+} ions in cell homogenates. Our results are in contrast with those of Newell and coworkers [Europe-Finner and Newell, 1985; Small et al., 1986] observed for saponin-permeabilized cells. They find that Ca^{2+} activates guanylate cyclase. The reason for this difference is unknown. Obviously, guanylate cyclase behaves differently in saponin-permeabilized cells and in electropermeabilized cells. Newell and coworkers also found that $InsP_3$ has a stimulatory effect on the enzyme, whereas we do not observe any effect in electropermeabilized cells. Furthermore, saponin-permeabilized cells do not show adaptation-like phenomena. The cause of these differences is unknown. It may be due to the different ways in which the

cell membrane is affected by the different treatments. In higher eukaryotes both inhibitory and stimulatory effects of Ca^{2+} on cGMP synthesis have been reported [Kimura and Murad, 1975; Lad and White, 1979; Europe-Finner and Newell, 1985; MacNeil, 1987; Klumpp et al., 1987; Koch and Stryer, 1988; Garbers, 1989, 1994; Garbers et al., 1994]. It remains to be seen which of these in vitro effects of Ca^{2+} reflect physiologically relevant in vivo regulatory events.

Summarizing, electroporabilized cells are a useful system to investigate the chemotactic cGMP response in *Dictyostelium* cells. Particularly, molecular processes that are related to adaptation and deadaptation can be investigated in this system under near-in vivo conditions.

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