THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 261, No. 32, Issue of November 15, pp. 15121-15125, 1986
Printed in U.S.A.

Surface Receptor-mediated Activation of Adenylate Cyclase in *Dictyostelium*

REGULATION BY GUANINE NUCLEOTIDES IN WILD-TYPE CELLS AND AGGREGATION DEFICIENT MUTANTS*

(Received for publication, May 8, 1986)

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GTP and GTP analogs produced significant (up to 17-fold) and persistent activation of adenylate cyclase in lysates of *Dictyostelium discoideum* amoeba. The activation was enhanced 2- to 4-fold by cAMP (the agonist for receptor-mediated adenylate cyclase activation), was specific for guanine nucleoside triphosphates, and was inhibited by guanosine 5′-(*O*-2-thio)diphosphate. The order of potency of guanine nucleotides was guanosine 5′-(*O*-3-thio)triphosphate > guanyl-5′-yl imidodiphosphate > GTP; half-maximal activation was observed with 1–10 μM guanine nucleotide. Maximal activation occurred when the guanine nucleotide was added within seconds after cell lysis and the lysate was preincubated for 5 min prior to assay. Under these optimal *in vitro* conditions, the capacity of guanine nucleotides to activate decreased, closely correlating with adaptation or desensitization induced by exposure of intact cells to cAMP during a period of 10 min. These data strongly support that regulation of adenylate cyclase in *Dictyostelium* occurs via a receptor-linked GTP/GDP exchange protein. Two mutants, designated *synag 7* and *49* were isolated in which cAMP and/or guanine nucleotides were not sufficient to activate adenylate cyclase. The wild-type pattern of guanine nucleotide regulation was restored to *synag 7* lysates by the addition of a high-speed supernatant from wild-type cells. Characterization of these mutants demonstrates that activation of adenylate cyclase is not required for growth or cell-type specific differentiation but is essential for cellular aggregation and influences morphogenesis and pattern formation. This suggests that *Dictyostelium* may provide a model suitable for detailed genetic analysis of surface receptor-guanine nucleotide-binding regulatory protein linked adenylate cyclase systems and for determining the role of these systems in development.

In vertebrate cells, surface receptor-linked adenylate cyclase systems involve three classes of membrane proteins: surface receptors (R), a catalytic component (C), and guanine nucleotide-binding regulatory proteins (G-proteins).[1] Receptors regulate catalytic activity by controlling the amount of the active, or GTP-bound, form of the G-protein. The G-proteins have a GTP/GDP exchange activity that is stimulated by agonist-occupied surface receptors and GTPase activity that is blocked by cholera toxin catalyzed ADP-ribosylation (1, 2). The G-protein family includes those which mediate stimulation (G_s) and inhibition (G_i) of the catalytic component and those whose functions remain undefined (G_o). The elegant biochemical studies which have led to these conclusions have been accompanied by few genetic studies. Several mutants of S49 lymphoma cells which are deficient or defective in G_s have been isolated but a detailed genetic analysis of receptor-linked adenylate cyclase systems has not been possible in mammalian cells (3, 4).

In yeast, where an extensive genetic analysis can be carried out, the discovery of guanine nucleotide regulation of adenylate cyclase initially promised to yield new insights into this transmembrane signaling system. However, GTP regulation of adenylate cyclase in yeast was found to be mediated by the *ras* proto-oncogene product rather than a heterotrimeric G-protein as in vertebrates (5, 6). Endogenous *ras* apparently does not regulate adenylate cyclase in vertebrates (7). Furthermore, there is no evidence that a surface receptor is coupled to the adenylate cyclase in yeast. *Dictyostelium* offers an alternative model system which is accessible to genetic analysis and contains a surface cAMP receptor which mediates activation of adenylate cyclase (reviewed in Refs. 8 and 9). This cyclase activation and the subsequent secretion of the newly synthesized cAMP are part of an intercellular communication system which emerges during development and coordinates a highly organized cellular aggregation. This receptor/adenylate cyclase system displays reversible agonist-mediated activation/desensitization properties that are analogous to the hormone and neurotransmitter-regulated adenylate cyclase systems in vertebrates (8).

Due to its accessibility both at the biochemical and genetic level and the extensive physiological characterization that is available, the receptor-linked adenylate cyclase system in *Dictyostelium* provides a useful model for comparison with vertebrate systems. However, previous attempts to demonstrate GTP stimulation of adenylate cyclase in these cells have been unsuccessful (8). We reasoned that the components

* This work was supported by National Institutes of Health Grant GM-28007 and a National Institutes of Health Biomedical Research Support Grant (to P. N. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ An American Heart Association Investigator.

[1] The abbreviations used are: G-protein, guanine nucleotide regulatory binding protein of adenylate cyclase; GTPγS, guanosine 5′-(*O*-3-thio)triphosphate; GDPβS, guanosine 5′-(*O*-2-thio)diphosphate; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; PTT, dithiothreitol; SDS, sodium dodecyl sulfate; PEI, polyethyleneimine; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; development buffer, 5 mM Na_2HPO_4, 5 mM KH_2PO_4, 2 mM $MgSO_4$; 200 μM $CaCl_2$.

TABLE I

Relative adenylate cyclase activity

AX-3 cells were lysed in the presence of the compounds indicated and preincubated at 0 °C for 5 min and then assayed for 1 min at 22 °C. Each value indicates the activity in the presence of the combination of the compounds listed in its row and column. Guanine nucleotides were 50 μM, cAMP was 1 μM, and Mn^{2+} was 5 mM. In the presence of Gpp(NH)p plus cAMP, the average activity was 125 ± 48 pmol/min/mg, n = 14. This assay was included in every experiment and all other activities were expressed as a percent of that in Gpp(NH)p plus cAMP so that the data from independent experiments could be pooled. Averages and standard deviations from at least five independent experiments for each determination are shown.

	No additions	cAMP	GDPβS	GDPβS plus cAMP	Mn^{2+}	Mn^{2+} plus cAMP	GDPβS plus Mn^{2+}
No additions	4.0 ± 1.7	16.7 ± 5.1	2.7 ± 0.9	3.4 ± 1.1	14.9 ± 4.4	ND[a]	11.9 ± 3.2
GTP	9.6 ± 2.5	46.7 ± 19.9	ND	ND	ND	ND	ND
Gpp(NH)p	36.8 ± 17.9	100	2.5 ± 0.7	13.5 ± 7.2	ND	14.0 ± 3.3	ND
GTPγS	70.2 ± 25.0	128 ± 28.8	ND	ND	ND	ND	ND

[a] ND, not determined.

might be rapidly uncoupled after cell lysis rendering the cyclase unresponsive to GTP *in vitro*. We report here that addition of GTP or GTP analogs within seconds after cell lysis followed by preincubation of the lysate with the nucleotide yields significant and persistent activation of the enzyme. The dependence of this activation on cAMP and GTP indicate that it is mediated by a GTP/GDP exchange protein that is influenced by the surface cAMP receptor. Having demonstrated the feasibility of studies of G-protein regulated receptor-linked adenylate cyclase in this organism, we show that mutants can be readily isolated and used to further define the components of this transmembrane signaling system.

EXPERIMENTAL PROCEDURES

RESULTS[2]

Activation by Guanine Nucleotides—The effect of various agents on adenylate cyclase activity in lysates is shown in Table I. GTP and two nonhydrolyzable GTP analogs, Gpp(NH)p and GTPγS, increased activity 2.4-, 9.2-, and 17-fold, respectively (column 1). Exposure of cells to cAMP for a few seconds at 0 °C before lysis stimulated activity 4.2-fold and enhanced the guanine nucleotide-dependent activity between 1.5- and 4-fold (column 2). In the presence of cAMP plus GTP, Gpp(NH)p, or GTPγS, activations were 11.7-, 25-, and 32-fold over basal, respectively. GDPβS did not activate and strongly inhibited the effects of Gpp(NH)p, cAMP, and cAMP plus Gpp(NH)p (columns 3 and 4). In the presence of GDPβS, these activities were reduced to 0.62-, 0.85-, and 3.4-fold of basal. These data indicate that the activation is specific for guanine nucleoside triphosphates and is influenced by occupied surface cAMP receptors. In the presence of Mn^{2+}, activity was not enhanced by addition of Gpp(NH)p plus cAMP and was only slightly reduced by GDPβS (columns 5–7). This is similar to the effect seen in vertebrate systems in which Mn^{2+} uncouples G-protein regulation of adenylate cyclase (1).

Fig. 2 shows that the concentration of guanine nucleotide which produced half-maximal stimulation was between 1 and 10 μM. Concentrations as low as 0.5 μM were effective in activation. The order of potency of the analogs was GTPγS > Gpp(NH)p > GTP. The maximal effect of GTP was significantly less than that of the nonhydrolyzable analogs. The effects of cAMP were maximal at 1 μM (data not shown). Gpp(NH)p had no effect on the K_m of the cyclase for ATP (K_m = 40 μM); the entire stimulatory effect could be attributed to an increase in the V_{max} of the enzyme (data not shown).

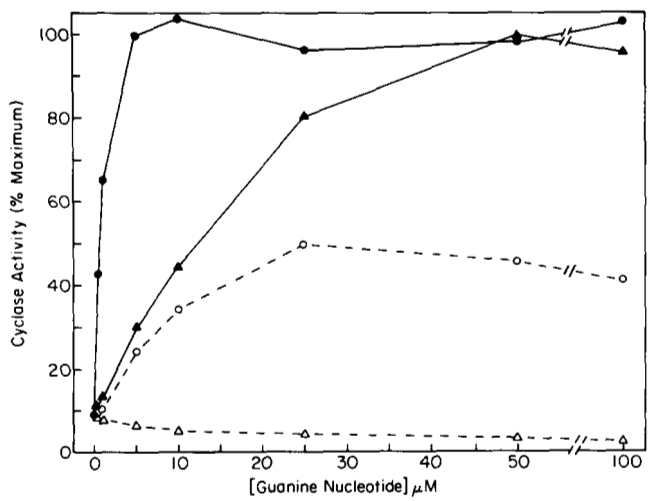

FIG. 2. **Concentration dependence of guanine nucleotide-dependent activations and inhibition.** Lysates were prepared in the presence of 1 μM cAMP plus the concentration of nucleotide indicated, preincubated for 5 min at 0 °C, and assayed for 1 min at 22 °C. The guanine nucleotides used were: GTP (○), Gpp(NH)p (▲), GTPγS (●), and GDPβS (△). 100% activity corresponds to an average of 123 ± 49 pmol/min/mg, n = 5.

Effect of Gpp(NH)p in Vitro following in Vivo Activation—The effects of persistent cAMP stimulation of intact cells on adenylate cyclase activity *in vitro* are shown in Fig. 3. Cells were lysed at increasing times after stimulation by cAMP and assayed for adenylate cyclase. Cells lysed in the absence of Gpp(NH)p were assayed either immediately after lysis or following a preincubation for 5 min at 0 °C. In the samples assayed immediately, persistent stimulation of intact cells with cAMP transiently activated the cyclase. The activity rose to a maximum at 1 min and fell to prestimulus levels after 5 min of cAMP stimulation. The elevated activity was unstable *in vitro*; in the lysates which were preincubated for 5 min at 0 °C the activation was nearly lost.

A stable activation was achieved if cells were lysed in the presence of Gpp(NH)p and preincubated for 5 min at 0 °C prior to assay. In lysates prepared prior to cAMP stimulation of the cells, Gpp(NH)p stimulated activity 9-fold. A few seconds of exposure of intact cells to cAMP enhanced the

[2] Portions of this paper (including "Experimental Procedures," part of "Results," and Fig. 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-1443, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

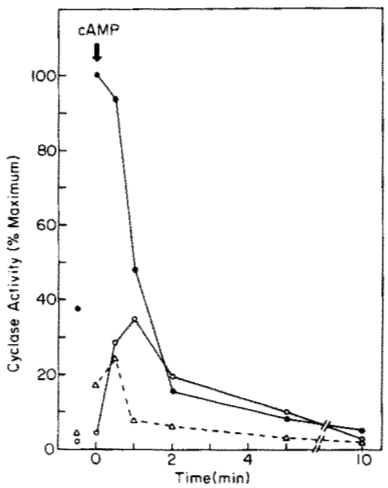

FIG. 3. **The effect of cAMP stimulation of intact cells on adenylate cyclase activation.** Lysates were prepared prior to and at the indicates times following stimulation of intact cells with 10 μM cAMP plus 10 mM dithiothreitol to inhibit degradation of cAMP by phosphodiesterase (indicated by *arrow*). Lysates were assayed immediately (○) or following preincubation for 5 min in the absence (△) or presence (●) of 50 μM Gpp(NH)p. Assays were for 1 min at 22 °C. 100% activity was 132 ± 49 pmol/min/mg, $n = 6$.

TABLE II

Mutants in adenylate cyclase system

Lysates were assayed and data analyzed as described in the legend to Table I. 100% activity = 129 ± 64 pmol/min/mg, $n = 4$. Wild-type cells were NC-4 strain.

Addition during lysis or assay	Source of lysate		
	Wild-type	*Synag 49*	*Synag 7*
	relative cyclase activity		
None	3.6 ± 1.4	3.5 ± 1.8	3.0 ± 1.0
Gpp(NH)p plus cAMP	100	6.6 ± 3.5	6.6 ± 3.1
$MnSO_4$	29.6 ± 13.9	11.6 ± 7.0	41.8 ± 27.3

effects of Gpp(NH)p by 3-fold (Table I, Fig. 3). Thereafter, the continued stimulation of intact cells with cAMP diminished the capacity of the enzyme to be activated *in vitro* by Gpp(NH)p. This effect was biphasic. There was an initial decline to about 20% of the maximal activity which occurred with a half-time of 1 min. A slower decline occurred between 2 and 10 min. After 10 min of persistent stimulation of intact cells with cAMP, the cAMP plus Gpp(NH)p activation attained *in vitro* was reduced to 5% of the maximum.

Cyclase Activation in Mutants—In order to investigate the role of adenylate cyclase during aggregation, we have studied the biochemistry of two aggregation defective mutants, *synag 49* and *synag 7*. Both of the mutants express normal surface cAMP receptors and demonstrate normal cAMP-stimulated cell shape change responses, cGMP accumulation, and chemotaxis.[3,4] Each can synergize with wild-type amoeba to sporulate, indicating that later development can proceed as long as the cells receive the appropriate extracellular signals. However, extracellular cAMP does not stimulate cAMP secretion in these two mutants. Neither mutant showed significant cAMP-activated adenylate cyclase activity in lysates assayed immediately after lysis. In addition, inclusion of Gpp(NH)p under conditions which lead to maximal activation of wild-type cyclase also did not produce significant activation in either of the mutants (Table II). The defect in *synag 49* may be related to the catalytic component of the adenylate cyclase since the Mn^{2+}-dependent activity was also reduced 3-fold (and as much as a 6-fold reduction has been observed). However, in *synag 7*, Mn^{2+}-dependent activity is at least as high as in wild-type suggesting that the defect is related to the regulatory component(s) of the cyclase.

We reasoned that the two lysates might complement *in vitro* since the biochemical phenotypes of the two mutants differed. When the lysates of *synag 49* and *synag 7* were mixed and preincubated with cAMP plus Gpp(NH)p, significant activation of adenylate cyclase was attained. This reconstitution occurred in the absence of detergents, which suggested that a soluble component was responsible for the reconstitution. The data in Table III show that a 100,000 × *g* supernatant fraction prepared from *synag 49* or wild-type amoeba restores Gpp(NH)p-dependent activation in *synag 7* lysates. The following additional properties of the reconstitution assay were consistently observed: 1) supernatants of *synag 7* were ineffective in reconstitution (Table III) but did not influence the Gpp(NH)p-dependent activation of wild-type lysates (not shown). 2) The cyclase activity in *synag 7* lysates assayed in the presence of Mn^{2+} was not influenced by addition of the wild-type supernatant (Fig. 4*A*). 3) The component of the supernatant which restored activation was inactivated by heating to 90 °C for 5 min and also inactivated by tryptic digestion. 4) The reconstitution was reversed by dilution or when *synag 7* membranes were separated from the wild-type supernatant. 5) Preincubation of *synag 7* lysates but not the donor wild-type supernatant with Gpp(NH)p was required for reconstitution. In addition, *synag 7* membranes prepared from

TABLE III

Reconstitution of synag 7 mutant

Buffer or supernatants from the indicated cell types were mixed with lysates of *synag 7* in the presence of Gpp(NH)p plus cAMP; the ratio of supernatant to *synag 7* lysate was 1:1. The mixture was preincubated for 10 min at 0 °C and then assayed for 1 min. 100% activity = 119 ± 46 pmol/min/mg, $n = 8$.

Buffer (no supernatant)	Source of supernatant		
	Synag 7	*Synag 49*	Wild-type
	relative cyclase activity		
5.7 ± 1.9	5.4 ± 2.3	46.4 ± 7.6	100

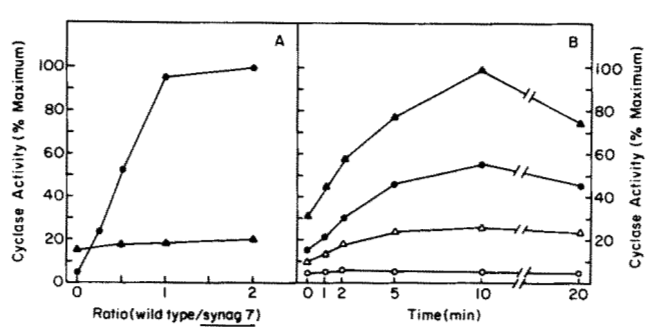

FIG. 4. **Reconstitution of wild-type phenotype to *synag 7* mutant.** *A*, the effect of wild-type supernatant on cyclase activity in *synag 7* lysates. *Synag 7* lysates were added to an equal volume of buffer and/or wild-type supernatant to achieve the supernatant/lysate ratio indicated, lysates were then preincubated at 0 °C for 10 min and assayed at 22 °C for 1 min. Activity in the absence (●) or presence of Mn^{2+} (▲). 100% activity = 148 ± 43 pmol/min/mg, $n = 5$. *B*, time course of reconstitution at different supernatant/lysate ratios. *Synag 7* lysates were added to an equal volume of buffer and/or wild-type supernatant at time = 0 min and preincubated at 0 °C for the time indicated before a 1-min assay at 22 °C. The supernatant/lysate ratios were: *O* (○), 0.25 (△), 0.5 (●), and 1.0 (▲). 100% activity = 106 ± 28 pmol/min/mg, $n = 5$.

[3] A. Theibert and P. N. Devreotes, unpublished observations.

[4] P. Van Haastert, personal communication.

synag 7 lysates preincubated with Gpp(NH)p could be reconstituted with wild-type supernatants prepared in the absence of Gpp(NH)p. 6) Gpp(NH)p-preincubated *synag 7* lysates assayed for several minutes in the absence of wild-type supernatant retained the capacity to be reconstituted by wild-type supernatant.

The dose dependence of reconstitution of *synag 7* lysates with wild-type supernatant is shown in Fig. 4*A*. The reconstitution saturated at a wild-type supernatant/*synag 7* lysate ratio (cell/cell) of 1. Activity of *synag 7* lysates assayed in the presence of Mn^{2+} was unaffected by addition of supernatant. The extent but not the kinetics of activation depended on the amount of supernatant added. Fig. 4*B* demonstrates that at each supernatant/lysate ratio, maximal activation was reached between 5 and 10 min of preincubation and a slight decrease between 10 and 20 min was observed.

DISCUSSION

Several lines of evidence have previously suggested that G-proteins are involved in transmembrane signaling in *Dictyostelium*: 1) GTP and GDP induce a decrease in the affinity of [^{3}H]cAMP binding in membranes (18), which is a characteristic effect of guanine nucleotides on the affinity of agonists for G-protein-linked receptors (19). 2) High concentrations (> 50 μM) of GTP and Gpp(NH)p noncompetitively inhibit cyclase activity in *Dictyostelium* membranes (20). 3) Cholera toxin catalyzes ADP-ribosylation and [^{32}P]8-N_3-GTP specifically photoaffinity labels a protein of similar molecular weight ($M_r = 44{,}000$) as the α subunit of G_s found in vertebrate cells. On two-dimensional gels, this protein and $G_s\alpha$ run similarly (21, 22). Although these results have suggested the presence of a G-protein in *Dictyostelium*, the data presented in this report are the first to demonstrate the specific *activation* of adenylate cyclase *in vitro* by guanine nucleotides.

Three conditions are required to observe maximal activation by Gpp(NH)p. These requirements may explain why previous attempts to activate the enzyme *in vitro* by GTP or GTP analogs were unsuccessful. First, the nucleotide must be added within a few minutes after cell lysis. The G-protein may be progressively uncoupled from the receptor following cell lysis and only those G-proteins remaining coupled to receptor can be "loaded" by GTP. Alternatively, the G-protein may be degraded during the first few minutes after lysis unless GTP (or analog) is bound to it. Second, the lysate must be preincubated for several minutes with the nucleotide prior to assay. The kinetics of activation at 0 °C may represent binding of GTP to the G-protein, or the interaction of receptor, regulatory protein(s), and catalytic component. A similar build-up in guanine nucleotide-dependent activation is observed in vertebrate adenylate cyclase systems (23). Third, a few seconds of *in vivo* exposure to cAMP is required for receptor-mediated enhancement of the guanine nucleotide activation. The effects of this brief cAMP stimulation persist *in vitro* since the enhancement is observed when Gpp(NH)p is added up to 5 min after cell lysis (Fig. 1).

Previous reports have shown that a transient cAMP-stimulated activation of adenylate cyclase in *Dictyostelium* can be observed if cAMP-stimulated cells are assayed immediately following lysis (24) (Fig. 2, *open circles*). This type of assay presumably reflects the state of activation of the enzyme *in vivo* at the moment of assay. The highest activity is observed in samples taken 1 min after exposure of intact cells to cAMP. The rapid *in vitro* decay of this activity ($t_{1/2} = 7$ s) may reflect the hydrolysis of GTP bound to the G-protein at the moment of lysis. In contrast, the stable guanine nucleotide-dependent activity we report here likely reflects the capacity of the enzyme to be activated *in vitro* rather than the *in vivo* activation which can be measured at the moment of lysis. The capacity to be activated by GTP is highest prior to or within a few seconds of exposure of cells to cAMP and progressively declines thereafter. The decline in activity represents the adaptation or desensitization that has been extensively investigated in *Dictyostelium* and a number of vertebrate cells. This adaptation process depends on continuous occupation of the surface receptor by the agonist cAMP. In *Dictyostelium*, receptors become progressively and extensively phosphorylated during persistent cAMP stimulation of intact cells (25). Modified receptors may be ineffective in loading GTP onto the G-protein. Alternatively, adaptation may take place at the level of the G-protein rendering it unable to interact with the catalytic subunit. The assays established here will be useful in determining the step at which adaptation takes place.

The mutant *synag 7* demonstrates that receptor-adenylate cyclase coupling is required for cellular aggregation. Cyclase activation does not appear to be essential for growth or cell-type specific differentiation, although pattern formation is altered in *synag 7*.[5] *Dictyostelium* may, therefore, be an ideal system for isolation of mutants in the receptor/G-protein-mediated regulation of adenylate cyclase.

In *synag 7* receptors and basal cyclase are apparently normal but addition of cAMP and/or guanine nucleotides is not sufficient to produce activation of adenylate cyclase *in vivo* or *in vitro*. Addition of a high speed supernatant fraction of wild-type lysates can restore the capacity of Gpp(NH)p to activate the *synag 7* enzyme. The donor supernatant could contain a soluble G-protein, although soluble G-proteins in connection with adenylate cyclase have not been widely recognized. The following observations suggest that the G-protein is present in *synag 7* membranes. First, preincubation of the *synag 7* lysate with Gpp(NH)p is required prior to reconstitution, while the wild-type donor supernatant need not be preincubated with Gpp(NH)p. Second, guanine nucleotides lower the affinity of [^{3}H]cAMP binding to a similar extent in *synag 7* and wild-type membranes.[4]

There have been previous reports of soluble components which enhance guanine nucleotide-dependent cyclase regulation (1, 26). The reconstitution of the *synag 7* mutant suggests that receptor-mediated activation of adenylate cyclase requires a fourth, soluble component in addition to the membrane bound receptor, G-protein, and catalytic components. The absence of this component (or its function) in *synag 7* and the capacity for reconstitution by wild-type supernatants opens the possibility for the identification and purification of this molecule.

Acknowledgments—We thank Peter Klein, Dr. Roxanne Vaughan, Dr. Maureen Pupillo, and Jane Borleis for reading this manuscript.

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Supplement to

Surface Receptor Mediated Activation of Adenylate Cyclase in Dictyostelium discoideum: Regulation by Guanine Nucleotides in Wild-Type Cells and Aggregation Deficient Mutants

Anne Theibert and Peter Devreotes

Experimental Procedures

Cell Growth and Development

Dictyostelium discoideum AX-3 cells were grown in HL-5 medium (10) to a density of 5 x 10^6/ml. washed in DB, resuspended at 2x10^7 cells/ml and shaken at 110-130 rpm for 4 to 6 hours. NC-4 cells and mutants synag 7 and synag 49 were isolated and grown as described (11,12) and were developed in suspension in DB at 2x10^7 cells/ml at 120 rpm and pulsed every 10 min with 50 nM cAMP for 12 hours. After development, cells were diluted to a density of 5x10^6 cells/ml with development buffer (DB): 5 mM Na_2HPO_4, 5mM KH_2PO_4, 2 mM $MgSO_4$, 200 uM $CaCl_2$ at 22° and shaken for 30 min at 200 rpm at 22°. The cells were then centrifuged at 600xg for 5 min at 0°, washed once with DB minus $CaCl_2$ at 0°, centrifuged and resuspended at 4x10^7/ml in DB without $CaCl_2$ at 0° . Adenylate cyclase was assayed in lysates prepared from cells shaken at 0° for at least 10 min and no longer than 60 min. This protocol ensured that extracellular cAMP was removed and that cells were unable to undergo spontaneous cAMP synthesis and secretion (13,14).

Adenylate Cyclase Assay

200 ul of cells at 4x10^7/ml at 0° were rapidly pipetted into a 1 ml tuberculin syringe containing 200 ul of 2x lysis buffer (20 mM Tris pH 8.0 and 2 mM $MgSO_4$) at 0° and were immediately pushed through two 5 um nucleopore filters (15) fitted between the barrel of the syringe and the needle (21g, 1.5 in). The lysate was collected into a tube at 0°. 200 ul of lysate was added to 20 ul of 10X reaction mix (1 mM ATP, 100 mM DTT, 1 mM cAMP and [α-^{32}P]-ATP in 200 mM Tris pH 8.0) and incubated for 1 min at 22°. The final composition of the reaction was: 4X10^6 cells (0.2 mg protein); 30 mM Tris pH 8.0, 2-10 X 10^6 cpm [α-^{32}P]-ATP; 2 mM $MgSO_4$, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 0.1 mM ATP, 10 mM DTT (a phosphodiesterase inhibitor), and 0.1 mM cAMP. The final concentration of guanine nucleotide, when included, was 0.5-100 uM. The final concentration of cAMP, when included during cell lysis, was 1 uM. "Unregulated" cyclase activity was measured by addition of 5mM $MnSO_4$ to the reaction mixture (1). The reaction was stopped with 100 ul of 1% SDS, 9mM ATP, 1mM cAMP. The reaction mix was diluted to 1 ml with H_2O and [^{32}P]cAMP was purified (16). The radioactivity was detected by Cerenkov counting in a Beckman scintillation counter. When 0.1 mM cAMP and 10 mM DTT were present in a 1 min reaction, less than 10% hydrolysis of exogenous [^{3}H]cAMP occurred during the reaction. Furthermore, an ATP regeneration system was omitted since less than 20% of the [α-^{32}P]ATP was degraded in the 1 min assay as monitored by separation of ^{32}P-labeled adenine nucleotides by PEI (Machory-Nagel MN 300, 0.1mm cellulose) chromatography.

Preparation of Supernatants

Wild-type and mutant cells were developed as described above. Cells were resuspended at 1X10^8 /ml at 0° in 10 mM Tris pH 8.0, 1mM $MgSO_4$, 0.2 mM EGTA, plus either 200 mM sucrose or 10% glycerol and lysed by passage through two 5 uM Nucleopore filters. The lysate was centrifuged at 20,000 X g for 10-30 min. The supernatant was removed and centrifuged at 100,000 X g for 60 min. High-speed supernatants were frozen at -70° and thawed immediately before reconstitution.

Reconstitution Assay

Synag 7 lysates were prepared by filter lysis as described in the adenylate cyclase section. Cells at a final density of 4-5 X 10^7/ml were lysed in the presence of 50 uM Gpp(NH)p plus 1 uM cAMP (final concentrations). After a 5 min preincubation at 0°, the lysate was adjusted to 10% glycerol and the lysates were divided into aliquots, frozen using an ethanol/dry ice bath and stored at -70°. Glycerol was required to preserve all of the cyclase activity after thawing frozen lysates. In the reconstitution assay, 120 ul of synag 7 lysate was thawed on ice and immediately added to 120 ul of buffer and/or recently thawed supernatant to the desired supernatant/lysate ratio. The mixture was incubated at 0° for the indicated time, then 200 ul of the mixture was added to 20 ul of 10X reaction mix (see above) and incubated at 22° for 1 min. The final dilution of the synag 7 lysate represented an equivalent of 2-2.5X10^7 cells/ml. Since the wild-type supernatant was prepared from cells at 1X10^8/ml, supernatant/lysate (cell/cell) ratios up to 2:1 could be utilized. In the trypsinization experiment, the supernatant was treated with 10 ug/ml of trypsin for 60 min at 0°; then 100 ug/ml soybean trypsin inhibitor was added and the mixture incubated for an additional 60 min at 0° before the reconstitution assay. In the control, the supernatant was incubated with buffer for 60 min at 0° and then soybean trypsin inhibitor was added prior to the trypsin and incubated for an additional 60 min at 0°.

Data Analysis

Variations in absolute adenylate cyclase activity were related to differences in the development of the amoebae and the amount of endogenous extracellular cAMP. In order that data from different experiments could be pooled, the following analysis was included in each experiment: Cells were lysed in the presence of cAMP plus Gpp(NH)p. The lysate was preincubated for 5 min at 0° and then incubated in reaction mix at 22° for 1 min. Thus, the activity measured in other assays is expressed as a percentage of the activity (100%) measured in this standard assay. The absolute average cyclase activity in these controls is given for each set of pooled data. Activity in the presence of Mn^{++} varied between experiments ranging from 10 and 70 % of the maximum. However, activity in the presence of Mn^{++} was never influenced by the addition of guanine nucleotides, cAMP, or supernatants.

Materials

cAMP, ATP, DTT, trypsin, and soybean trypsin inhibitor were purchased from Sigma. GTP and Gpp(NH)p were purchased from Boehringer Mannheim or Sigma. GDP-β-S and GTP-δ-S were obtained from Boehringer Mannheim. [α-^{32}P]ATP was synthesized according to the method of Johnson and Walseth (17).

Results

Requirements for Activation by Guanine Nucleotides

Figure 1 presents the conditions required for maximal stimulation by guanine nucleotides. Preincubation in vitro at 0° is required to achieve maximal activation by Gpp(NH)p, cAMP, and Gpp(NH)p plus cAMP (Figure 1A). Cells were lysed in the presence of these compounds, then preincubated for increasing times at 0° prior to a 1 min assay at 22°. In lysates which were assayed immediately, the cyclase activation was only 5 to 25 % of the maximum. The activation increased during the initial 5 min of preincubation at 0°; the half-time for the build-up in activity was between 1 and 2 min. Basal activity (in the absence of Gpp(NH)p or cAMP) also increased about 2-fold after 5 min at 0° (from 2 to 4 pmols/min-mg). GDP-β-S inhibited this increase in basal activity (Table 1), which suggests that this increase may be due to low levels of endogenous cAMP and GTP. Therefore, the activity observed in the presence of GDP-β-S likely reflects the true basal activity. The data in Figure 2 also illustrate that the Gpp(NH)p-dependent activated state is stable for at least 10 min at 0°. The slight decrease in cAMP and cAMP plus Gpp(NH)p stimulated activity which occurred between 5 and 10 min of preincubation appeared to be due to a loss of catalytic activity since the activity in the presence of Mn^{++} also decreased after 5 min (data not shown). Addition of 10% glycerol to the lysate prevented any loss of activity for at least 15 min, but reduced the overall activation about 2-fold (not shown). The Gpp(NH)p dependent activation was also stable to freezing if the lysates were prepared in glycerol.

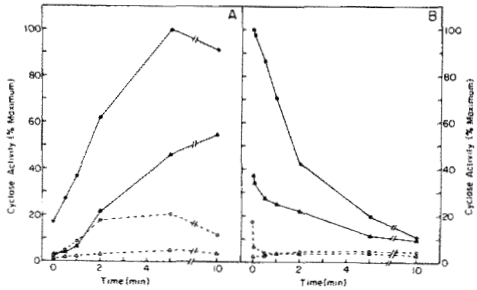

Figure 1. Requirements for optimal guanine nucleotide activation. A. Time course of adenylate cyclase activation by Gpp(NH)p and/or cAMP. Lysates were prepared in the absence (△) or in the presence of 1 uM cAMP (○), 50 uM Gpp(NH)p (▲) or 50 uM Gpp(NH)p plus 1 uM cAMP (●). The lysate was preincubated at 0° for the time indicated and then assayed for 1 min at 22°. 100% activity corresponds to an average activity of 130 ± 45 pmols/ min-mg protein n=7. B. Time course of the loss in stimulation by Gpp(NH)p or cAMP. Cells were lysed and the lysate incubated at 0° for the time indicated prior to the addition of Gpp(NH)p or cAMP. The lysates were incubated for an additional 5 min at 0° and assayed for 1 min at 22°. Additions were: none (△), 1 uM cAMP (○), 50 uM Gpp(NH)p (▲), 50 uM Gpp(NH)p added to lysates prepared in the presence of 1 uM cAMP (●). 100% activity was 135 ± 55 pmols/min-mg protein n=6. For t=0, the cells were lysed in the presence of the indicated compound.

Figure 1B shows that the capacity of Gpp(NH)p to activate adenylate cyclase in vitro was highest at the moment of cell lysis and declined when the lysates were preincubated in its absence. In this experiment, Gpp(NH)p, cAMP, or cAMP plus Gpp(NH)p were present either during lysis or were added to the lysate at later times. Each lysate was then preincubated for an additional 5 min at 0° before assay (as in Figure 1A). Maximal activation was attained when cells were lysed in the presence of cAMP plus Gpp(NH)p. However, when cells were lysed in the presence of cAMP and Gpp(NH)p was added within a few seconds, a comparable high activation (97%) was observed. Addition of Gpp(NH)p to the lysate at later times was less effective; the loss of the capacity to activate occurred with a half-time of approximately 2 min. Ten min after lysate preparation, addition of Gpp(NH)p produced only a 2-3 fold stimulation over basal activity. Although the capacity of cAMP alone to stimulate activity was lost immediately after lysis, the presence of cAMP during lysis continued to enhance the effects of Gpp(NH)p addition for up to 10 minutes of incubation at 0°.