Proc. Nat. Acad. Sci. USA
Vol. 71, No. 8, pp. 3087–3090, August 1974

5′-Guanylylimidodiphosphate, A Potent Activator of Adenylate Cyclase Systems in Eukaryotic Cells

(hormones/membranes/cyclic AMP/guanine nucleotides/allosteric regulation)

CONSTANTINE LONDOS*, YORAM SALOMON*, MICHAEL C. LIN*, JAMES P. HARWOOD*, MICHAEL SCHRAMM*, J. WOLFF†, AND MARTIN RODBELL*

* Section on Membrane Regulation, Laboratory of Nutrition and Endocrinology, and † Section on Endocrine Biochemistry, Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Maryland 20014

Communicated by E. R. Stadtman, May 22, 1974

ABSTRACT 5′-Guanylylimidodiphosphate (Gpp(NH)-p) stimulates adenylate cyclase [ATP-pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity in plasma membranes isolated from frog and salmon erythrocytes, from rat adrenal, hepatic, and fat cells, and from bovine thyroid cells. The nucleotide acts cooperatively with the various hormones (glucagon, secretin, ACTH, thyrotropin, and catecholamines) that stimulate these adenylate cyclase systems with resultant activities that equal or exceed those obtained with hormone plus GTP or with fluoride ion. In the absence of hormones, Gpp(NH)p is a considerably more effective activator than GTP, and, under certain conditions of incubation, stimulates rat fat cell adenylate cyclase to levels of activity (about 20 nmoles of 3′,5′-adenosine monophosphate mg protein per min) far higher than reported hitherto for any adenylate cyclase system examined. The nucleotide activates frog erythrocyte adenylate cyclase when the catecholamine receptor is blocked by the competitive antagonist, propranolol, and activates the enzyme from an adrenal tumor cell line which lacks functional ACTH receptors. In contrast, Gpp(NH)p does not stimulate adenylate cyclase in extracts from *Escherichia coli* B. Gpp(NH)p appears to be a useful probe for investigating the mechanism of hormone and nucleotide action on adenylate cyclase systems in eukaryotic cells.

Several studies have shown that GTP enhances the response of adenylate cyclase systems [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] to peptide hormones, catecholamines, and prostaglandins (1–6). Kinetic studies of the hepatic glucagon-sensitive adenylate cyclase system indicate that GTP mediates the actions of glucagon on the enzyme system by an interdependent process indicative of an allosteric enzyme system (7). 5′-Guanylylimidodiphosphate (Gpp-(NH)p)‡, the terminal phosphate of which appears not to be utilized in phosphotransferase reactions (8), mimics the stimulatory effects of GTP (9). In this paper we report that Gpp(NH)p causes marked stimulation of adenylate cyclase activity in membranes isolated from eukaryotic cells irrespective of the nature of the receptors coupled to these systems and even in the absence of hormones. Activation by the nucleotide occurs by means of a time-dependent process that is accelerated by hormones. In all cases, the effects of Gpp(NH)p are greater than those of GTP. Combinations of Gpp(NH)p and hormones produce activities considerably greater than those seen in the presence of fluoride ion; the latter agent, with few exceptions, has been thought previously to stimulate maximally most adenylate cyclase systems (10).

Shown in Fig. 1 are typical effects of epinephrine, fluoride ion, and Gpp(NH)p on the time course of adenylate cyclase activity in purified plasma membranes from rat adipocytes. Fluoride ion and epinephrine, at maximally effective concentrations, elicited immediate stimulatory effects on enzyme activity. In contrast, 1 μM Gpp(NH)p increased activity after a lag of 1 min of incubation. This lag was diminished by addition of the various hormones (secretin, glucagon, ACTH, and epinephrine) that activate the fat cell adenylate cyclase system (data not shown). The lag in onset of activation by Gpp(NH)p, and the effects of hormones thereon, is typical of all adenylate cyclase systems examined in this study. As will be shown in detailed studies to be published later, the initial inhibitory effect of the nucleotide shown in Fig. 1 is abolished when the pH of the incubation medium is increased from 7.6 to 8.5. The latter pH is optimal for the fat cell system.

It will be noted in Fig. 1 that the steady state rates achieved with 1 μM Gpp(NH)p are approximately 2-fold higher than observed with 10 mM fluoride ion. Under optimal incubation conditions (1 mM ATP, 50 mM Mg^{2+}, pH 8.5, and 37°) for the fat cell adenylate cyclase system, 0.1 mM Gpp(NH)p stimulates activity to approximately 20 nmoles of cyclic AMP per min/mg of membrane protein (Fig. 2). This value, obtained in the absence of hormones, is the highest activity given by any of the adenylate cyclase systems examined in this study and is only slightly elevated by addition of all the hormones that affect the fat cell system.

A comparison of the effects of maximal levels of GTP and Gpp(NH)p on the hepatic adenylate cyclase system is shown in Table 1. While GTP increased basal activity by 20%, the increase with Gpp(NH)p was 350%, resulting in a rate approaching that seen with GTP plus glucagon. As was observed with the fat cell enzyme system, activation of the hepatic enzyme system by Gpp(NH)p was preceded by a lag period. For example, with a submaximal concentration of Gpp(NH)p (50 nM), the lag was as long as 3–4 min, and was reduced to 1 min by the addition of glucagon. This phenomenon is of interest in view of the previous finding with the hepatic adenylate cyclase system that GTP abolishes the lag in onset of activation by low concentrations of glucagon (7). It appears that both Gpp(NH)p and GTP mediate the action of the hormone through a common site or process. However,

Abbreviations: Gpp(NH)p, 5′-guanylylimidodiphosphate; cyclic AMP, 3′,5′-adenosine monophosphate; $ACTH^{1-24}$, tetracosactide 1–24 corticotropin; DTT, dithiothreitol.

‡ Supplied by International Chemical and Nuclear Corp.

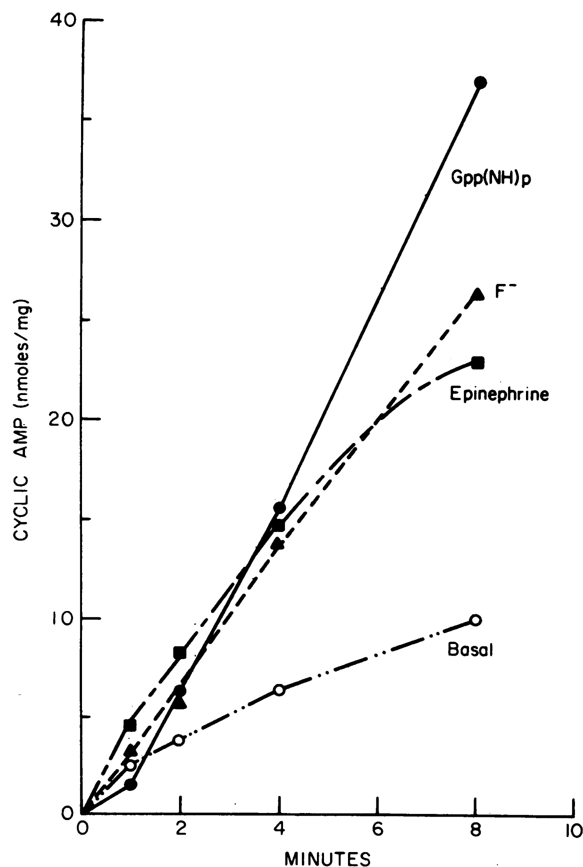

FIG. 1. Effects of epinephrine, fluoride ion, and Gpp(NH)p on fat cell adenylate cyclase activity. Purified plasma membranes were isolated from fat cells of the rat by previously described methods (9, 15). In these experiments, fat cell membranes (11 μg of protein per ml) were incubated at 37° in media containing 1 mM [α-^{32}P]ATP, 40 mM $MgCl_2$, ATP regenerating system consisting of 10 mM creatine phosphate and 50 units of creatine kinase, 25 mM Tris·HCl, pH 7.6, 1 mM dithiothreitol (DTT), 2.5 mM cyclic AMP and 0.2% bovine serum albumin. The concentration of Gpp(NH)p was 1 μM; NaF, 10 mM; and epinephrine 0.35 mM. With fat cell membranes, and with all other membrane preparations examined in this report, the reaction was stopped and [^{32}P]cyclic AMP was isolated according to a recently described procedure (16).

Gpp(NH)p alone activates the enzyme by a time-dependent process suggestive of a slow transition between different states of the enzyme; this transition appears to be accelerated in the presence of hormones and guanine nucleotides.

Previous studies have shown the thyrotropin-sensitive adenylate cyclase system from bovine thyroid cells to be only minimally responsive to GTP (3). However, as is seen in Table 2, Gpp(NH)p increased basal activity 12-fold to give a rate approaching that observed with fluoride ion and considerably greater than that seen with thyrotropin. Indicative of the cooperative interaction of hormone and nucleotide on the thyroid enzyme system is the observation that the combination of Gpp(NH)p and thyrotropin resulted in an activity greater than the sum of activities obtained when each ligand was tested separately.

Table 3 shows a comparison of the effects of Gpp(NH)p, ACTH, and fluoride ion on adenylate cyclase activity in membranes prepared from rat adrenal and from two mouse adrenal tumor cell lines: Y-1, which is responsive to ACTH, and

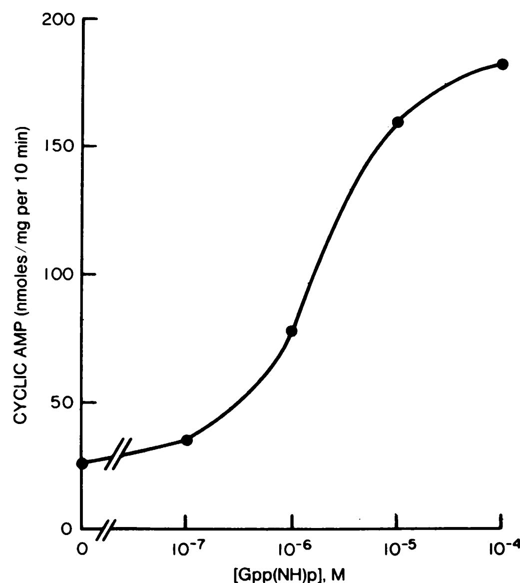

FIG. 2. Concentration dependency of Gpp(NH)p-stimulation of fat cell adenylate cyclase activity. Fat cell membranes (10 μg of membrane protein per ml) were incubated for 10 min at 37° in medium containing 1 mM [α-^{32}P]ATP, 50 mM $MgCl_2$, 1 mM DTT, 2.5 mM cyclic AMP, 0.2% bovine serum albumin, and 25 mM Tris·HCl, pH 8.5.

OS3, which does not respond to the hormone (11). The enzyme from rat adrenal was activated by Gpp(NH)p, and the nucleotide acted cooperatively with the hormone to increase activity to a level considerably greater than that observed with fluoride ion; activation of basal and hormone-stimulated activities by Gpp(NH)p was twice that observed with GTP (data not shown). The enzyme system from the ACTH-responsive tumor cell line, Y-1, also exhibited the cooperative interaction of nucleotide and hormone, whereas that from the OS3 cell line, while completely unresponsive to hormone, was activated by Gpp(NH)p. Thus, stimulation by Gpp(NH)p occurs even in the apparent absence of a functional hormone receptor.

It will be noted in Table 3 that, while little difference was observed between fluoride-stimulated activity in the two adrenal tumor cell enzymes, basal and Gpp(NH)p-stimulated activities were considerably lower with the enzyme from the

TABLE 1. *Effects of Gpp(NH)p, GTP, and glucagon on hepatic adenylate cyclase*

Additions	Adenylate cyclase activity pmoles of cAMP per min/mg of protein: Basal	Glucagon (1 μM)
None	8	33.6
GTP, 0.1 mM	9.8	39.2
Gpp(NH)p, 0.1 mM	31	50.4

Purified plasma membranes from rat liver were prepared as described previously (17). Membranes, 0.72 mg of protein per ml, were incubated at 30° for 5 min in medium consisting of 5 mM $MgCl_2$, 1 mM cAMP, and 25 mM Tris·HCl, pH 7.5. Substrate was 0.1 mM [α-^{32}P]5′-adenylylimidodiphosphate (1).

TABLE 2. *Adenylate cyclase activity in bovine thyroid membranes: effects of thyrotropin, Gpp(NH)p, and fluoride*

Additions	Adenylate cyclase activity (pmoles of cyclic AMP per mg of protein per min)		
	Basal	Thyrotropin	Fluoride
None	60	239	1060
Gpp(NH)p, 10 μM	729	1648	—

Thyroid membranes were purified as described previously (18). Adenylate cyclase assay medium contained 1 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 1 mM cyclic AMP, 5 mM phosphocreatine, 50 U/ml of creatine phosphokinase, and 25 mM Tris·HCl, pH 7.6, in a total volume of 0.1 ml. Reaction time was 10 min at 37° with the use of 0.16 mg/ml of membrane protein. Thyrotropin concentration was 200 mU/ml, and fluoride, 10 mM.

OS3 cells than that from the Y-1 cells. These differences appear to be related to incubation temperature. For example, at 30° Gpp(NH)p exerts little stimulatory effect on the enzyme from the OS3 cells but markedly stimulates the Y-1 enzyme. However, at 43° Gpp(NH)p-stimulated activity with the OS3 enzyme was somewhat greater than with the Y-1 enzyme. The basis of the differences in temperature sensitivity of the adenylate cyclase systems in OS3 and Y-1 adrenal tumor cells is under investigation.

Activation of adenylate cyclase systems was observed also in hormone-sensitive cells obtained from amphibia and fish. Table 4 shows the cooperative effects of Gpp(NH)p and isoproterenol on the frog erythrocyte adenylate cyclase system. Gpp(NH)p and isoproterenol increased activity 10- and 20-fold, respectively. When the nucleotide and the hormone were combined, the activity increased 60-fold and attained a rate three times higher than that produced with fluoride ion. Similar effects of Gpp(NH)p and isoproterenol were obtained with the adenylate cyclase system in purified plasma membranes from salmon erythrocytes.

Propranolol, an agent which blocks β-receptors in adenylate cyclase systems, inhibited the stimulatory effect of isoproterenol, but not that of Gpp(NH)p (Table 4). These findings demonstrate that Gpp(NH)p stimulates adenylate cyclase activity by a mechanism independent of receptor occupation, a conclusion reinforced by the observation that the nucleotide increased activity in an adrenal tumor cell line which lacks a functional receptor, and by the fact that Gpp(NH)p causes near maximal activation of the fat cell enzyme system in the absence of hormones.

Gpp(NH)p did not alter the activity of adenylate cyclase in extracts of *Escherichia coli* B under incubation conditions comparable to those employed with the eukaryotic adenylate cyclase systems. Adenylate cyclase in *E. coli* B appears to play an important regulatory role in the phenomenon of glucose repression of enzyme induction (12). The lack of effect of Gpp(NH)p on *E. coli* B adenylate cyclase suggests that regulation of bacterial and eukaryotic adenylate cyclase systems may differ with respect to their nucleotide requirements.

TABLE 4. *Activation of frog erythrocyte adenylate cyclase by Gpp(NH)p in absence and presence of isoproterenol: lack of inhibition by propranolol of Gpp(NH)p effect*

Additions	Adenylate cyclase activity pmoles of cyclic AMP per min/mg of protein
None	110
Fluoride	2450
Isoproterenol	2440
Isoproterenol + propranolol	110
Gpp(NH)p	1300
Gpp(NH)p + isoproterenol	6820
Gpp(NH)p + propranolol	1360
Gpp(NH)p + isoproterenol + propranolol	1360

Frog erythrocyte plasma membranes were prepared according to Rosen and Rosen (20) with the following modifications. After cell lysis, membranes obtained from 100 ml of frog blood were sedimented by centrifugation for 20 min at 12,000 × *g*. The pellet was suspended in 40 ml of Tris·HCl buffer, pH 8.1, containing 1 mM DTT, and centrifuged at 24,000 × *g* for 10 min. The pellet was washed twice as described above and stored in the Tris·DTT buffer under liquid nitrogen at a concentration of about 5 mg of protein per ml. Adenylate cyclase activity was measured under the same incubation conditions described in *Table 2* for the thyroid adenylate cyclase system. Incubations were carried out with 33 μg of membrane protein per ml for 5 min at 30°. Final concentration of isoproterenol, propranolol (Sigma), and Gpp(NH)p was 10 μM. Fluoride ion was 10 mM.

TABLE 3. *Adenylate cyclase activity in membranes from rat adrenal tumor cells: effects of ACTH$^{1-24}$, Gpp(NH)p, and fluoride*

Enzyme source	Adenylate cyclase activity (pmoles of cyclic AMP per mg of protein per 5 min)				
	Basal	Gpp(NH)p	ACTH$^{1-24}$	Gpp(NH)p + ACTH$^{1-24}$	NaF
Rat adrenals	144	708	957	2468	1466
Adrenal tumor cells					
Y-1 cells	34	163	155	437	1065
OS3 cells	13	93	16	94	925

Adrenals from 150 g male Sprague–Dawley rats and mouse tumor cells grown in monolayer (11, 19) were homogenized with six strokes in a ground glass homogenizer in 250 mM sucrose, 1 mM DTT, 20 mM Tris·HCl, pH 7.6. Homogenates were centrifuged at 600 × *g* for 10 min, and the pellets suspended in 20 mM Tris·HCl, pH 7.6, 1 mM DTT, and stored under liquid nitrogen. Adenylate cyclase activity was measured under the same incubation conditions described in *Table 2* for the thyroid adenylate cyclase system. Reaction time was 5 min at 37° with the use of approximately 0.2 mg/ml of membrane protein. The concentration of Gpp(NH)p was 10 μM, ACTH$^{1-24}$ 1 μM, and fluoride 20 mM. ACTH$^{1-24}$ (Synacthen) was a gift of CIBA-Geigy. Y-1 cells were obtained from the American Type Culture Collection; we gratefully acknowledge the gift of the OS3 cells by Dr. B. P. Schimmer.

It has been suggested recently (7) that glucagon regulates hepatic adenylate cyclase activity by inducing the formation of states of the enzyme system that have increased reactivity with GTP. 5′-Guanylyldiphosphonate (Gpp(CH_2)p) (1) and Gpp(NH)p, analogs with γ-phosphates that are not readily cleaved, substitute for GTP in the activation of the enzyme. Therefore, it is reasonable to suggest that guanine nucleotides do not serve as phosphate donors but as allosteric activators of adenylate cyclase systems. The present study shows that Gpp(NH)p is a potent activator of eukaryotic adenylate cyclase systems, irrespective of the nature of the receptor coupled to these system; the nucleotide is an effective activator in the absence of hormones, and even in the absence of functional hormone receptors.

Activation by Gpp(NH)p is a time-dependent process. Hormones appear to facilitate the rate at which Gpp(NH)p activates the enzyme systems, as evidenced by the finding that addition of hormones diminishes the lag in onset of the Gpp(NH)p effect. In studies to be reported elsewhere, increasing the reaction temperature also diminishes the lag in onset of activation by Gpp(NH)p. These findings suggest that the process of adenylate cyclase activation may proceed through different transition states which equilibrate slowly in the absence of hormone; addition of hormone or increasing the reaction temperature permits more rapid equilibration between these states. This view is in accord with the concepts proposed by Ainslie *et al.* (13), and by Frieden (14), to explain transient kinetic characteristics of a number of regulatory enzymes. Whether such changes involve associative reactions between subunits remains to be determined for the complex, membrane-bound adenylate cyclase systems.

The basis for the differences in effectiveness of GTP and Gpp(NH)p is unknown. It is possible that the γ-phosphate of GTP is hydrolyzed at the activation site, and that the rate of hydrolysis of GTP may be faster than the rate of transition between the various states suggested above. Gpp(NH)p, in contrast, being resistant to hydrolysis, may remain bound to the activation site. Studies to be reported elsewhere will show that adenylate cyclase systems remain activated following pretreatment with Gpp(NH)p and extensive washing to remove all but bound nucleotide, and that resultant enzyme activity corresponds closely to the amount of nucleotide bound to the membranes.

In conclusion, this report shows that Gpp(NH)p stimulates adenylate cyclase activity in plasma membranes obtained from a variety of eukaryotic cells. Gpp(NH)p appears to act through the same process by which GTP mediates hormone action; however, under appropriate incubation conditions, the analogue may stimulate the enzyme systems to maximal catalytic capacity even in the absence of hormones. The cooperative interactions of hormones and nucleotides, originally discovered by the use of GTP, demonstrate the importance of the nucleotide function in hormonal regulation of adenylate cyclase systems. The finding that Gpp(NH)p activates in the absence of hormones suggests that the nucleotide provides a potentially powerful tool for investigating the processes by which hormones and nucleotides activate adenylate cyclase systems in eukaryotic cells.

Dr. Londos was supported by a National Institutes of Health Fellowship (4 F03 DE51657-03) from the National Institute of Dental Research. Dr. Schramm is on sabbatical leave from the Hebrew University, Jerusalem, Israel.

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