Altered Guanine Nucleotide Hydrolysis as Basis for Increased Adenylate Cyclase Activity after Cholera Toxin Treatment

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Cholera toxin activation of the adenylate cyclase of mouse neuroblastoma cells occurs in situ with intact cells or in vitro with cell membranes. The in vitro activation process requires NAD and nucleotide triphosphates, in addition to toxin. The cholera toxin-activated adenylate cyclase, when assayed at physiological concentrations of MgCl₂ (10 mM) in assay systems containing ATP and a nucleotide-regenerating system, has a very high activity and is not significantly activated by 2-chloroadenosine, prostaglandin E₁, guanyl-5'-yl imidodiphosphate, or NaF. Under similar assay conditions, the activity of the normal enzyme is low, and maximal activity requires the above activators. The normal enzyme after activation by guanyl-5'-yl imidodiphosphate closely resembles the toxin-activated enzyme when assayed at 10 mM MgCl₂. However, these two enzymes can be distinguished from each other. When the MgCl₂ concentration exceeds 20 mM, the toxin-activated enzyme, but not the guanyl-5'-yl imidodiphosphate-activated enzyme, again requires exogenously added guanine nucleotide triphosphates, NaF, or prostaglandin E₁, for maximal activity.

By using adenylyl-5'-yl imidodiphosphate as substrate (in assays with and without a nucleotide-regenerating system) it was found that the high activity of the toxin-activated enzyme observed at 10 mM MgCl₂ requires the presence of guanine nucleotide triphosphates. GTP or guanyl-5'-yl imidodiphosphate can fulfill this requirement. The source of the endogenous guanine nucleotides in the assays which contain ATP and a regenerating system appears to be the membrane preparation itself. The normal enzyme can only use these endogenous guanine nucleotides as activators in the presence of 2-chloroadenosine or prostaglandin E₁. 2-Chloroadenosine and prostaglandin E₁ do not activate the normal enzyme in the absence of guanine nucleotides. Analysis of the interaction of guanyl-5'-yl imidodiphosphate and GDP with the normal and toxin-activated enzyme reveals no significant differences between the two enzymes.

We propose that at physiological concentrations of MgCl₂ the toxin-activated enzyme is "fixed" in a highly active state (E-GTP). The normal enzyme decays from such a GTP state rapidly at physiological concentrations of MgCl₂. Activation of the enzyme by cholera toxin results in a reduction or prevention of this decay mechanism. A general model of control of the activity of adenylate cyclase by guanine nucleotides, 2-chloroadenosine, prostaglandin E₁, as well as other enzyme activators, is presented.

Cholera toxin, an enterotoxin produced by Vibrio cholerae, produces its diarrheagenic effect by activating the adenylate cyclase (EC 4.6.1.1; ATP pyrophosphate-lyase (cyclizing)) of the small intestine (1-5). The original suggestion that adenylate cyclase was activated by cholera toxin was made by Michael Field (5). Cholera toxin has been observed to stimulate adenylate cyclase in a wide variety of intact cells and tissues to which it has been added (see review, Ref. 6). Based on the work of King and Van Heyningen (7) and confirmed by others (8-13), such a wide range of effectiveness appears to be due to the fact that the primary recognition site on the cell for cholera toxin is the ubiquitous G₃₃ ganglioside in mammalian plasma membranes.

The cAMP concentration of tissues or isolated cells can be increased by specific hormones or cholera toxin (1, 14-16). When the adenylate cyclase from toxin-treated cells is assayed in vitro, it exhibits greatly elevated activities in the absence of any hormonal stimulators (17-24). Many of these toxin-activated enzymes have been found to show altered responses to hormones, guanine nucleotides, and MgCl₂ (17, 19, 24). However, it remains unclear which, if any, of these alterations are responsible for the increased activity displayed by these enzymes.

The mechanisms regulating the activity of the normal adenylate cyclase have also been difficult to elucidate. We have reported that increases in the cAMP concentration in intact mouse neuroblastoma cells can be elicited by adenosine and prostaglandin E₁ (25). The normal adenylate cyclase of these cells, when assayed in vitro, is activated by adenosine, certain adenosine analogues, i.e. 2-chloroadenosine (26), PGE₁ (27), guanyl-5'-yl imidodiphosphate (28), and NaF (27, 28). Based on the in vitro effects of various combinations of the above effectors, we have proposed that guanine nucleotides (GTP and GDP) are the primary regulators of the activity of adenylate cyclase. PGE₁ and adenosine were considered secondary enzyme regulators since they were found to control the primary regulatory process (28). Efforts to solubilize an unaltered...
envelope from the plasma membranes of various cells, including the neuroblastoma cells, have not been successful. Because of the limitation of working with the normal adenylate cyclase in its membrane environment, a new approach was sought to confirm and further extend the previous novel proposals for regulation of adenylate cyclase. The studies reported here, on the characterization of the toxin-activated adenylate cyclase from mouse neuroblastoma cells, confirm the primary role of guanine nucleotides in regulation and suggest that cholera toxin alters this primary recognition process.

**Materials and Methods**

**Growth of Cells**—All studies were performed on clonal line NS20 of mouse neuroblastoma Cl300 (29). Cells were maintained as stock cultures in Falcon flasks and grown in glass roller bottles for large preparations (26). The growth media was Dulbecco's Modified Eagle's Medium supplemented with 10% horse serum, penicillin G (10 units/ml), and streptomycin sulfate (10 μg/ml). Cells were treated with cholera toxin by replacing the growth medium of confluent cells with an equal volume of serum-free medium containing 20 μg/ml of cholera toxin and incubating at 37°C for various times up to 18 h (see text).

**Preparations of Adenylate Cyclase**—Cells were harvested by shaking without the use of trypsin and were washed and homogenized as previously described to produce a crude homogenate (26). Unless otherwise indicated, a particulate fraction was isolated from the crude homogenate by centrifugation at 600 × g for 10 min at 4°C, followed by centrifugation of this supernatant at 37,000 × g for 30 min at 4°C. The resultant pellet was resuspended in 10 mM Tris/maleate buffer, pH 7.4, and could be frozen and stored in liquid nitrogen without loss of activity for at least 6 months. The enzyme prepared from cells which were not treated with toxin will be referred to as normal enzyme. Toxin-activated enzyme will refer to the enzyme prepared from cells which were incubated with 20 μg/ml of toxin for 18 h, unless noted otherwise.

**Adenylate Cyclase Assays**—Production of cAMP was assayed at 30°C using either α-[32P]ATP or α-[32P]AMP-P(NH)P according to the method of Solomon et al. (30) with modifications as previously described (26). Unless otherwise specified in the text, assays with cAMP were performed with 50 mM Tris/maleate buffer, pH 7.4, 1 mM ATP, 6 mM MgCl₂, 0.5 mM cAMP, 0.3 mM RO20-1724 (cAMP phosphodiesterase inhibitor), and an ATP-regenerating system consisting of 20 mM creatine phosphate and 0.1 mM sodium ATP. Assays with AMP-P(NH)P were as above but do not contain a regenerating system (unless specified in text) and contain 0.2 mM AMP-P(NH)P. Proteins were determined by the method of Lowry et al. (31). All specific activities are reported as pmoles of cAMP produced/min/mg of protein. All activity values, unless otherwise specified, were averages of duplicate determinations, varying by less than 15%.

**Materials**—Cholera toxin was purchased from Schwarz/Mann. ATP, GDP, GTP, creatine phosphokinase, creatine phosphate, and CIAdo were purchased from Sigma Chemical Co. GMP-P(NH)P and AMP-P(NH)P were purchased from P-L Biochemicals, Inc. [α-32P]ATP was obtained from ICN Corp. (50 to 100 Ci/mmol) and New England Nuclear (20 to 25 Ci/mmol). [α-32P]AMP-P(NH)P (7 to 12 Ci/mmol) was purchased from ICN Corp. RO20-1724 and PGE₁ were gifts from Hoffmann-La Roche, Inc., Nutley, N. J.

**Results**

**Activation of Adenylate Cyclase in Situ by Cholera Toxin**—Incubation of intact NS20 with cholera toxin leads to increased "basal" adenylate cyclase (e.g. activity measured with homogenates at 10 mM MgCl₂) in the absence of stimulators (Fig. 1). The effects of the various known enzyme activators and inhibitors on the activity of the toxin-activated enzyme and normal enzyme have been compared (Table 1). The activity of the normal adenylate cyclase is elevated by CIAdo, PGE₁, GMP-P(NH)P, and NaF. None of these compounds significantly elevates the activity of the enzyme isolated from cells treated with toxin for 18 h. The activity of the toxin-activated enzyme is equal to the activity of the fully stimulated normal enzyme. The increases in "basal" activity and the decreases in the activation produced by the above activators are not seen immediately after addition of toxin, but are subject to a characteristic lag of 1 h. (2, 8, 32-34). Both aspects of the in situ activation processes are virtually complete within 2 h after addition of toxin.

The neuroblastoma enzyme, like other adenylate cyclases (35-38), possesses a regulatory site which is specific for gua...
nine nucleotides (28). GMP-P(NH)P, an analogue of GTP which is not readily converted to GDP (39), activates the normal enzyme at 10 mM MgCl₂ (Table I). In contrast, GMP-P(NH)P does not stimulate the toxin-activated enzyme at 10 mM MgCl₂. GDP and GTP alone do not influence the activity of either enzyme. An increase in the activity of the toxin-activated enzyme, but not the normal enzyme, is observed when GTP is added in combination with PGE₁. The same qualitative difference between the activity of the normal and toxin-activated enzyme is observed when the concentration of ATP in the assay is either 1.0 or 0.1 mM (data not shown).

Effects of Mg²⁺, Mn²⁺, and Cu²⁺—Mg²⁺ appears to be required along with ATP to form the active metal:ATP substrate complex for the neuroblastoma (27) and other adenylate cyclases (40). Moreover, Mg²⁺ influences the rate of activation of the normal enzyme by GMP-P(NH)P (28). Mg²⁺ also affects the guanine nucleotide regulatory site of the toxin-activated enzyme (Fig. 2). The activity of the toxin-activated enzyme is progressively inhibited when MgCl₂ exceeds 20 mM. Addition of GMP-P(NH)P prevents any loss of activity at high MgCl₂ concentrations and actually results in higher activity than is seen in the absence of GMP-P(NH)P at lower MgCl₂ concentrations. At 100 mM MgCl₂, the activity of the toxin-activated enzyme, when assayed with GMP-P(NH)P, is between 6- and 10 fold higher than when assayed without GMP-P(NH)P. At 100 mM MgCl₂, the Kₘ for this effect of GMP-P(NH)P on the toxin-activated enzyme is 2.9 ± 1 μM (data not shown). Concentrations of Mg²⁺ in excess of 20 mM produce other striking changes in the toxin-activated enzyme (Table I). At 100 mM MgCl₂, the toxin-activated enzyme and the normal enzyme have comparable low "basal" activities. Both are stimulated by PGE₁ and NaF, as well as GMP-P(NH)P. Addition of GTP or GDP (resulting in an equal mixture of GTP and GDP in the assay under these conditions) elevates the activity of the normal enzymes less than 2-fold and blocks activation of this enzyme by GMP-P(NH)P. In contrast, the GTP/GDP mixture stimulates the toxin-activated enzyme almost as much as does GMP-P(NH)P, and the GTP/GDP mixture only slightly inhibits the activation of GMP-P(NH)P.

For the normal enzyme, Mn²⁺ can replace Mg²⁺ in the active metal:ATP substrate complex (27). The MgCl₂ and MnCl₂ dose-response curves for both the normal and toxin-activated adenylate cyclases are quite similar (Fig. 3). With both enzymes, a bell-shaped activity curve is seen in the absence of GMP-P(NH)P. GMP-P(NH)P prevents the decline in activity with increasing divalent cation concentration for both enzymes, although with Mn²⁺ the protection is not complete. The inhibition of the toxin-activated enzyme caused by high concentrations of MgCl₂ and MnCl₂ does not appear to be due to a nonspecific increase in the ionic strength of the assay mixture. Addition of 200 mM sodium or ammonium chloride does not cause any inhibition (Table II). CaCl₂ will cause an inhibition of activity which is not prevented by GMP-P(NH)P (see below).

The activation of the normal neuroblastoma enzyme by GMP-P(NH)P appears to be irreversible. Similar findings

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**Fig. 2.** Effect of MgCl₂ on the activity of the toxin-activated enzyme. Activity was assayed with 1 mM ATP without GMP-P(NH)P (○) and with 100 mM GMP-P(NH)P (•).

**Fig. 3.** Comparison of normal and toxin-activated enzymes at various concentrations of Mg²⁺ or Mn²⁺. Assays were performed in the ATP assay system with 1 mM ATP with either MgCl₂ (Panels A and C) or MnCl₂ (Panels B and D). Normal enzyme assayed without GMP-P(NH)P (○) and with 100 μM GMP-P(NH)P (□). Toxin-activated enzyme assayed without GMP-P(NH)P (•) and with 100 μM GMP-P(NH)P (□). G, GMP-P(NH)P. In each panel, maximal activity was determined from the 1/V versus 1/ion plots shown in the inset.

**TABLE II**

| Ions present in assay mixture | Adenylate cyclase activity (pmol/min/mg protein) |
|------------------------------|-----------------------------------------------|
| MgCl₂ (10 mM)                | 32.8 ± 3.6                                    |
| MgCl₂ (100 mM)               | 16.8 ± 1.1                                    |
| MgCl₂ (50 mM) + NaCl (200 mM)| 31.9 ± 1.5                                    |
| MgCl₂ (10 mM) + NH₄Cl (200 mM)| 25.5 ± 2.5                                   |
| MgCl₂ (10 mM) + CaCl₂ (100 mM)| 1.5 ± 0.5                                   |
| MnCl₂ (1 mM)                 | 26.6 ± 5.0                                    |
| MnCl₂ (50 mM)                | 8.3 ± 0.9                                     |
have been reported with other adenylate cyclases (41, 42).

After incubation with GMP-P(NH)P and subsequent washing, the normal enzyme resembles the toxin-activated enzyme when assayed at 10 mM MgCl₂ (Table III); the activity of the GMP-P(NH)P-activated enzyme is maximal in the absence of any stimulators. PGE₁, Cl₄Ado, NaF, or GMP-P(NH)P do not stimulate activity any further and GDP, by itself or in the presence of GMP-P(NH)P, does not affect activity. CaCl₂ inhibits the activity of this enzyme (see below). The GMP-P(NH)P and toxin-activated enzyme, however, can be distinguished from each other. At 100 mM MgCl₂, the GMP-P(NH)P-activated enzyme, unlike the toxin-activated enzyme, is only slightly less active than it is at 10 mM MgCl₂. Furthermore, the GMP-P(NH)P-activated enzyme is not stimulated by either NaF, PGE₁, or GMP-P(NH)P at 100 mM MgCl₂.

CaCl₂ is an equally effective inhibitor of the normal and toxin-activated neuroblastoma adenylate cyclase. With the normal enzyme, CaCl₂ is noncompetitive with the enzyme substrate, Cl₄Ado (27), PGE₁, and GMP-P(NH)P (data not shown).

In all experiments with CaCl₂, the Kᵢ for calcium is the same, i.e. 340 μM. CaCl₂ inhibits the toxin-activated enzyme when assayed at low or high MgCl₂, with or without GMP-P(NH)P (Fig. 4), PGE₁, or Cl₄Ado with the same Kᵢ (data not shown).

Requirements for ATP for Normal and Toxin-Activated Enzymes—For the normal enzyme, the apparent Kᵢ for ATP increases as the MgCl₂ concentration increases (Fig. 5). Increases in the Kᵢ for ATP are also seen with the toxin-activated enzyme as the MgCl₂ concentration is increased. For the toxin-activated enzyme, these changes are more dramatic than those seen for the normal enzyme. With both enzymes, GMP-P(NH)P prevents a large part of these elevations in the Kᵢ (Fig. 5). For the toxin-activated enzyme, the MgCl₂ concentration at which the change in Kᵢ for ATP becomes dramatic corresponds to the concentration of MgCl₂ which begins to inhibit activity as well as allow stimulation of activity by GMP-P(NH)P.

Enzyme Stability—Several investigators have reported that activation by guanine nucleotides, NaF, and hormones stabilizes the activity of various adenylate cyclases (35, 43-46). We observe that increasing MgCl₂ concentration increases the decay rate of the toxin-activated enzyme (Fig. 6). Inclusion of GMP-P(NH)P not only prevents any loss in activity, but actually results in the recovery of an enzyme which is more active after incubation than before incubation. Although adding GMP-P(NH)P to a complete assay mixture which contains less than 10 mM MgCl₂ does not result in activation, adding GMP-P(NH)P to an incubation mixture containing only buffer and 10 mM MgCl₂ does result in a 50% increase in activity of the toxin-activated enzyme. The activity of the toxin-activated enzyme does not become more dependent upon added guanine nucleotides as the incubation period increases from 5 to 60 min. The following observations were made in similar experiments performed on the normal enzyme. (a) Decay is faster at higher MgCl₂ concentrations; (b) GMP-P(NH)P prevents decay; and (c) the decay rates of "basal" activity of the normal enzyme are not significantly different from those seen for the toxin-activated enzyme, at either low or high MgCl₂ concentrations.

Other nucleotides have been tested for their ability to affect the stability of the toxin-activated enzyme (Fig. 7). At 10 mM MgCl₂, decay is prevented by GTP or ATP when they are added in combination with the nucleotide-regenerating system. AMP-P(NH)P alone will also prevent decay, whereas, the regenerating system does not affect decay. Although ATP, GTP, and AMP-P(NH)P prevent decay at 10 mM MgCl₂, they do not result in the appearance of a more active enzyme. At 30 mM MgCl₂, only GMP-P(NH)P is completely effective in preventing enzyme decay. GTP, ATP, and AMP-P(NH)P, in this order, are only partially effective.

Studies with AMP-P(NH)P As Enzyme Substrate—We have reported previously (28) that AMP-P(NH)P can be used as a substrate by the normal adenylate cyclase from neuroblastoma cells. In an adenylate cyclase assay which contains 10 mM MgCl₂ and AMP-P(NH)P as substrate but which does not contain a nucleotide-regenerating system, the activity of the toxin-activated enzyme is similar to that of the normal enzyme (Table IV). The activity of both enzymes is very low; both are stimulated by NaF and GMP-P(NH)P, but neither are stimulated by PGE₁ or Cl₄Ado. When GTP is added in combination with PGE₁, stimulation of the toxin-activated enzyme occurs. The combination of GMP-P(NH)P and PGE₁ results in greater activity for the toxin-activated enzyme than is seen with GMP-P(NH)P alone. Addition of ATP, with or without PGE₁, does not affect the activity of either enzyme. The Kᵢ for GMP-P(NH)P for the toxin-activated and normal enzyme in this assay system is 1.3 ± 0.3 μM (data not shown) and 0.5 ± 0.2 μM (29), respectively.

The addition of the regenerating system to the AMP-P(NH)P assay system causes a 7-fold increase in the basal activity of the toxin-activated enzyme; no change in the activity of the normal enzyme is observed. Further, small increases in activity of the toxin-activated enzyme occur when PGE₁, PGE₂, plus ATP, PGE₂, plus GTP, or Cl₄Ado plus ATP are added in the presence of the regenerating system. In the presence of the regenerating system, Cl₄Ado and PGE₁ do stimulate the activity of the normal enzyme (28).

In an AMP-P(NH)P assay system devoid of a regenerating system (Table V), both enzymes exhibit the same activity at 100 mM MgCl₂ as they do at 10 mM MgCl₂, and both enzymes are stimulated by NaF and GMP-P(NH)P at 100 mM MgCl₂. The regenerating system does not stimulate the activity of

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**Table III**

Comparison of normal and GMP-P(NH)P-activated adenylate cyclase

| Assay additions | Normal enzyme | GMP-P(NH)P-activated enzyme |
|-----------------|---------------|-----------------------------|
| 5 mM MgCl₂      | 22.3          | 16.6                        |
| 5 mM MgCl₂      | 10.4          | 17.1                        |
| 100 mM MgCl₂    | 10.4          | 17.1                        |

**Adenylate cyclase activity**

- Normal enzyme
- GMP-P(NH)P-activated enzyme
- 5 mM MgCl₂
- 100 mM MgCl₂

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FIG. 4 (left). Noncompetitive interaction of Ca$^{2+}$ and GMP-P(NH)P with the toxin-activated adenylate cyclase. Assays were performed in the presence of 1 mM ATP and 100 mM Mg$^{2+}$. Activity was assayed with increasing concentrations of GMP-P(NH)P and the following amounts of CaCl$_2$, none (O), 0.2 mM (A), 0.05 mM (C), or 1 mM (D).

FIG. 5 (center). Changes in apparent ATP $K_m$ with increasing Mg$^{2+}$ concentrations. The concentration of ATP required for half-maximal enzyme activity at various Mg$^{2+}$ concentrations was determined for both the normal (O, D) and toxin-activated enzymes (A, A). Apparent ATP $K_m$ values were determined in the absence and presence of 100 mM GMP-P(NH)P. Normal without GMP-P(NH)P (O); normal with GMP-P(NH)P (D); toxin-activated without GMP-P(NH)P (A); and toxin-activated with GMP-P(NH)P (A).

FIG. 6 (right). Effect of Mg$^{2+}$ on the decay of the toxin-activated enzyme. The enzyme was incubated at 30°C for varying times in 10 mM Tris/maleate buffer, pH 7.4; and either 10 mM MgCl$_2$ (O, D); 100 mM MgCl$_2$ (A, A); or 100 mM MgCl$_2$ plus 100 mM GMP-P(NH)P (D). The activity remaining was then determined in the ATP assay system at 10 mM MgCl$_2$, 0.1 mM ATP; and with either no GMP-P(NH)P (O, O) or 100 mM GMP-P(NH)P (A, A). When GMP-P(NH)P was present in the incubation mixture, it was also present at 10 mM in the assays. Enzyme preparations were assayed directly after incubation. If after incubation, the enzyme is first washed twice before being assayed, the same results are obtained. Where the activities are assayed in the absence of GMP-P(NH)P, the initial activity is that seen without incubation. Where the activities are assayed with GMP-P(NH)P, the initial activity is that observed after a 10-min incubation with 10 mM Mg$^{2+}$ and 100 mM Mg-P(NH)P. Initial activities for normal and toxin-activated enzymes are 4.5 and 33.4 pmol/min/mg of protein, respectively.

Table IV: Comparison of normal and toxin-activated adenylate cyclase in AMP-P(NH)P assay system

| Assay additions | No. reg. system | Plus reg. system |
|-----------------|----------------|-----------------|
|                 | Normal | Toxin-activated | Normal | Toxin-activated |
| Adenylate cyclase activity | pmol/min/mg protein | pmol/min/mg protein |
| None            | 0.88   | 0.67            | 0.64   | 4.01           |
| ClAdo           | 0.38   | 0.60            | 1.04   | 3.70           |
| PGE$_1$         | 0.46   | 0.50            | 2.18   | 5.50           |
| NaF             | 8.70   | 4.90            | 2.91   | 5.90           |
| GTP             | 0.78   | 0.98            | 0.43   | 2.71           |
| GTP/ClAdo       | 0.74   | 1.20            | 0.81   | 3.60           |
| GTP/PGE$_1$     | 1.76   | 2.94            | 2.29   | 7.20           |
| ATP             | 0.50   | 0.30            | 3.22   |               |
| ATP/ClAdo       | 0.80   | 6.10            |        |               |
| ATP/PGE$_1$     | 0.81   | 0.40            | 7.40   |               |
| GMP-P(NH)P      | 3.50   | 4.71            |        |               |
| GMP-P(NH)P/ClAdo| 4.90   |                |        |               |
| GMP-P(NH)P/PGE$_1$ | 7.00  |                |        |               |

Either enzyme at 100 mM MgCl$_2$, yet GTP and ATP added together with the regenerating system selectively stimulates the activity of the toxin-activated enzyme. In the absence of a regenerating system, when AMP-P(NH)P is used as substrate,
TABLE V

Effect of high Mg\(^2+\) on enzyme activity in AMP-P(NH)P assay system

Assays were performed in the AMP-P(NH)P assay system with 0.9 mM AMP-P(NH)P at either 10 or 100 mM MgCl\(_2\) as noted. Other assay additions were as follows: ClAdo (100 

\mu M); PGE\(_2\) (1.4 

\mu M); Na\(\text{F}\) (5 mM); GTP (200 

\mu M); ATP (200 

\mu M); GMP-P(NH)P (100 

\mu M), and Reg. System, the nucleotide-regenerating system as described in the legend to Table IV.

| Assay additions | Normal | Toxin-activated |
|-----------------|--------|-----------------|
|                 | 10\(^{\circ}\) | 100\(^{\circ}\) | 10\(^{\circ}\) | 100\(^{\circ}\) |
| None            | 0.4 | 0.2 | 1.5 | 1.4 |
| ClAdo           |       |       |       |       |
| PGE\(_2\)       | 0.2 | 0.1 | 1.0 | 0.5 |
| Na\(\text{F}\)  | 11.6 | 8.7 | 4.9 | 2.9 |
| Reg. System     |       |       |       |       |
| Reg. System/GTP | 0.3 | 0.4 | 3.3 | 4.1 |
| Reg. System/GTP/ClAdo | 1.4 | 0.9 | 8.0 | 2.8 |
| Reg. System/ATP | 3.5 | 2.5 |       |       |
| Reg. System/ATP/ClAdo | 6.1 | 2.4 |       |       |
| Reg. System/ATP/PGE\(_2\) | 0.4 | 0.4 | 7.4 |       |
| GMP-P(NH)P      | 2.7 | 3.5 | 4.7 | 9.7 |
| GMP-P(NH)P/ClAdo | 4.9 | 9.4 |       |       |
| GMP-P(NH)P/PGE\(_2\) | 4.0 | 4.8 |       |       |

\^\text{a} \text{mM MgCl}_2.

PGE, and ClAdo increase the concentration of GDP required to block activation of the normal enzyme by GMP-P(NH)P (28). We find that 25 

\mu M GDP, which inhibits 50\% of the GMP-P(NH)P activation of the normal enzyme, also inhibits 50\% of the GMP-P(NH)P activation of the toxin-activated enzyme. Furthermore, PGE, increases the concentration of GDP required to block the activation of the toxin-activated enzyme by GMP-P(NH)P (Fig. 8).

In Vitro Activation of Adenylate Cyclase by Cholera Toxin—Recently, the successful in vitro activation of other adenylate cyclases by cholera toxin has been demonstrated. NAD, ATP, and cytoplasmic components of the cells, as well as dissociated cholera toxin, were reported to be necessary for the in vitro activation process (47-52). In general, the in vitro activation results in an enzyme with properties similar to those of the enzyme activated in situ by the toxin.

In vitro activation of the normal neuroblastoma enzyme occurs when a cell-free membrane preparation is incubated with cholera toxin (after treatment with dithiothreitol, NAD, and nucleotide triphosphates) (Table VI). Half-maximal activation occurs at 6.5 

\mu g/ml of toxin and 30 

\mu M NAD (data not shown). We do not observe any effect of the cytoplasm on this process. The requirement for a nucleotide is partially fulfilled by the inclusion of a nucleotide-regenerating system in the incubation mixture. Activation is maximal when ATP or GTP is added along with the regenerating system. In the absence of a regenerating system, GDP, ADP, GTP, ATP, or AMP-P(NH)P do not support the activation process (Table VI). The time course of the activation process in the complete incubation mixture has a \(t_{1/2}\) of 3 min (Fig. 9A). Based on the disappearance of stimulation by Na\(\text{F}\), the activation process appears to be complete after 20 min. Toxin activation will also take place during an adenylate cyclase assay if NAD is present (Fig. 9B). Under such conditions, the activation process has a similar \(t_{1/2}\) and after about 12 min the activity of the enzyme reaches a constant rate. The rate of the in vitro activation has
When AMP-P(NH)P is used as substrate in assays devoid of a nucleotide-regenerating system, the E-GTP state is converted to E-GDP, and with ATP as substrate, the basal activity of the normal enzyme assayed at 30° in 10 mM Tris/maleate buffer, pH 7.4, 10 mM MgCl₂, and either no NaF (○) or 5 mM NaF (●). B, activity of the normal enzyme assayed at 30° in 50 mM Tris/maleate buffer, pH 7.4, 10 mM MgCl₂, 1 mM ATP, regenerating system, 0.1 mM NAD, 0.1 mM NAD, plus the following additions: nothing (○); 50 μg/ml of choleran toxin (pretreated with dithiothreitol (○) or 5 mM NaF (●).

been noted by others (51) to be a function of the amount of free A₁ subunit of choleran toxin present. Dithiothreitol treatment of the toxin does not apparently fully dissociate the toxin as evidenced by the fact that additional treatment of the toxin with sodium dodecyl sulfate causes both the rate of in vitro activation and extent of activation of the neuroblastoma adenylate cyclase to increase (data not shown).

DISCUSSION

Previous studies on the normal neuroblastoma adenylate cyclase (28) led us to propose the model of enzyme regulation which is diagrammatically shown in Fig. 10. In our model, guanine nucleotides are the primary regulators of enzyme activity. When GTP or GMP-P(NH)P is bound to the guanine nucleotide site, the enzyme (E-GTP) or E-GMP-P(NH)P is in a state of high catalytic activity. When the guanine nucleotide site is occupied (E-0) or filled with GDP(E-GDP), the enzyme has little or no activity. GDP functions as an inhibitor by preventing the binding of the activator guanine nucleotides. When assayed at physiological concentrations of Mg²⁺ (10 mM) and with ATP as substrate, the basal activity of the normal neuroblastoma adenylate cyclase is low unless ClAdo, PGE₂, GMP-P(NH)P, or NaF is present. ClAdo and PGE₂ are secondary enzyme regulators and do not activate the enzyme. In the absence of guanine nucleotides. They cause an increase in activity by reducing the ability of GDP to prevent the binding of GTP or GMP-P(NH)P (28).

The studies reported here show that the interaction of choleran toxin with intact neuroblastoma cells or membranes isolated from these cells leads to an activation of adenylate cyclase. The toxin-activated enzyme exists, in the standard ATP assay system at physiological concentration of Mg²⁺ (10 mM), in a fully active state in the absence of ClAdo, PGE₂, NaF, or GMP-P(NH)P. According to our model, the toxin-activated enzyme should exist, under these assay conditions, in an E-GTP state in the absence of ClAdo, PGE₂, GMP-P(NH)P, or NaF. The primary source of the endogenous guanine nucleotide triphosphate in these assays appears to be the membrane preparation itself. When AMP-P(NH)P is used as enzyme substrate in assays devoid of a nucleotide-regenerating system but with 10 mM Mg²⁺, the toxin-activated enzyme and normal enzyme both have the same low "basal" activity. ClAdo and PGE₂ do not stimulate either enzyme, and both require the addition of GMP-P(NH)P or NaF in order to express maximal catalytic activity. Addition of a regenerating system to these assays results in the toxin-activated enzyme once again displaying, in the absence of ClAdo or PGE₂, an elevated catalytic activity as compared with the normal enzyme. In addition, there is now a return in the ability of PGE₂ and ClAdo to activate. We have observed that the amount of membrane protein routinely used in these assays contains sufficient phosphatase activity to completely convert 1 mM GTP to GDP within minutes in the presence of 10 mM MgCl₂ at 30°. It appears that the regenerating system contained in the standard ATP assays maintains the endogenous guanine nucleotides of the membrane preparations as triphosphates. However, only the toxin-activated enzyme has the ability to use them as activators in the absence of ClAdo or PGE₂.

We have found that at 10 mM MgCl₂, the toxin-activated enzyme state is similar to the state in which the normal enzyme exists at 10 mM MgCl₂ after incubation with GMP-P(NH)P. Washing the enzyme after GMP-P(NH)P activation does not reverse the process. The fact that GMP-P(NH)P, unlike GTP, cannot readily be converted to GDP implies that some type of metabolism of GTP, which GMP-P(NH)P cannot undergo, may be required for conversion of the E-GTP to the E-GDP. The GMP-P(NH)P-activated enzyme state, however, is not identical with the toxin-activated state. Elevation of the MgCl₂ concentration above 20 mM converts the toxin-activated enzyme, but not the GMP-P(NH)P-activated enzyme, to a state which again requires exogenously added guanine nucleotides or secondary enzyme regulators for maximal catalytic activity. High MgCl₂ inhibits the "basal" activity of the normal enzyme, and the MgCl₂ inhibition of both enzymes is prevented by GMP-P(NH)P. The most simple explanation for these findings is that the conversion of GTP to GDP at the enzyme's regulatory site is controlled by a Mg²⁺-dependent GTPase. Filling the guanine nucleotide site with GMP-P(NH)P yields an enzyme which is not inhibited by MgCl₂ as no conversion of GMP-P(NH)P to GDP is possible. The inhibition of both enzymes by MnCl₂, which we have observed is more complex. Some of the MnCl₂ inhibition occurs at the guanine nucleotide regulatory site as it is partially prevented by GMP-P(NH)P. However, MnCl₂ also inhibits by binding to the separate Ca²⁺ sites on the enzyme. Evidence for the fact that Ca²⁺ and Mn²⁺ can interact at the same site and thereby inhibit activity has been presented previously (27). Ca²⁺ and GMP-P(NH)P do not interact at the same sites on the enzyme.
and no change in the sensitivity of the enzyme to Ca\(^{2+}\) has been observed after toxin activation.

At physiological concentrations of Mg\(^{2+}\), the normal and toxin-activated enzymes have similar \(K_m\) values for ATP. As the MgCl\(_2\) concentration increases, there is an increase in the \(K_m\) for both enzymes. This increase is much more dramatic for the toxin-activated enzyme than for the normal enzyme. GMP-P(NH)P prevents most of the increase in apparent ATP \(K_m\) for both enzymes. This increase is much more dramatic for the toxin-activated enzyme than for the normal enzyme. GMP-P(NH)P will stabilize both enzymes at low Mg\(^{2+}\). An indirect effect of Mg\(^{2+}\) on the ATP-ase activity of the enzyme is due to the fact that this concentration of Mg\(^{2+}\) is most likely due to its influence on a different regulatory process, i.e. the affinity of GDP for the nucleotide regulatory site.

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