Cholera Toxin Activation of Adenylate Cyclase

ROLES OF NUCLEOSIDE TRIPHOSPHATES AND A MACROMOLECULAR FACTOR IN THE ADP RIBOSYLATION OF THE GTP-DEPENDENT REGULATORY COMPONENT*

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Keiichi Enomoto‡ and D. Michael Gill§
From the Department of Biology, Harvard University, Cambridge, Massachusetts 02138, and Department of Molecular Biology and Microbiology, Tufts University, Boston, Massachusetts 02111

Cholera toxin-catalyzed ADP ribosylation of the membrane-bound GTP-binding protein that regulates adenylate cyclase activity requires certain components of the cytosol. In broken cells it is prevented by depletion of endogenous nucleotides and is restored by the provision of GTP (1 to 3 μM half-maximum) or Gpp(NH)p (3 μM) although not by ATP, CTP, TTP, or UTP. An hydrolysis-resistant GTP analog, guanyl-5'-yl imidodiphosphate (Gpp(NH)p, 1 to 3 μM), also supports the toxin-catalyzed reaction, but adeny-5'-yl imidodiphosphate is only weakly effective. The ability of nucleoside triphosphates to support toxin-catalyzed ADP ribosylation or the toxin-catalyzed activation of adenylate cyclase correlate well.

Even with excess GTP or Gpp(NH)p, the rate of ADP ribosylation and adenylate cyclase activation depends upon the presence of a macromolecular component of the erythrocyte cytosol. If GTP is employed, ADP ribosylation requires the simultaneous presence of this nucleotide, cytosolic macromolecules, NAD⁺, toxin, and membranes. However, membranes may be predisposed to respond to the toxin by preincubating them with Gpp(NH)p and cytosolic macromolecules. Preincubated membranes, washed free of unbound nucleotide, can then be ADP ribosylated by incubation with cholera toxin and NAD⁺ without additional cofactors. Both Gpp(NH)p and GTP (1 μM or less) are required for such preactivation. It is complete in 15 min at 37°C and is about three times slower at 25°C. It requires magnesium, is accelerated by isopropenol, and is inhibited by GTP or sodium fluoride. The conditions for preactivation resemble those for the specific binding of Gpp(NH)p to the regulatory component of adenylate cyclase and consequent quasi-permanent activation of the cyclase, except that direct activation of the cyclase does not depend upon a cytosolic macromolecule.

We conclude that the substrate for cholera toxin is a complex of the GTP-binding protein with GTP, in some way modified by a macromolecule of the cytosol.

The exotoxin produced by Vibrio cholerae activates adenylate cyclase in many types of vertebrate cells (1). When supplied to intact cells the toxin binds to ganglioside GM₁ on the cell surface and subsequently releases its active fragment A₁ into the cytoplasm. The injected fragment A₁ catalyzes an intracellular reaction that causes irreversible activation of adenylate cyclase. Fragment A₁ alone activates adenylate cyclase in broken cell systems where toxin binding to cell membranes is neither required nor pertinent.

It has recently become clear that cholera toxin activates the cyclase by altering a property of the GTP-dependent regulatory component of the adenylate cyclase system (2-11). According to the model of Cassel and Selinger (12), the binding of GTP to the regulatory component of adenylate cyclase causes the formation of a ternary complex (GTP-regulatory component-catalytic component) that is catalytically active but, because of rapid hydrolysis of GTP to GDP, is ephemeral. Hence exogenous GTP alone results in little cyclase activity, but hydrolysis-resistant analogs of GTP elevate the activity greatly. β-Adrenergic agonists are thought to stimulate adenylate cyclase by increasing the rate of dissociation of GDP from the regulatory component so that the binding site can be reoccupied more rapidly by GTP.

Treatment of cells or membranes with cholera toxin elevates basal and hormonally stimulated adenylate cyclase activity (both measured with GTP) (2-6) by inhibiting the associated hydrolysis of GTP and allowing the activated state to persist longer (2, 3). The basis of this inhibition was recently shown to be the catalysis by cholera toxin of an ADP ribosylation of the GTP-dependent regulatory component itself (9, 10), a protein (M, 42,000) exposed on the inner face of the plasma membrane that had been previously identified by Pfeuffer (13) as the GTP-binding protein involved in the regulation of the activity of adenylate cyclase in pigeon erythrocyte membranes. Subsequent work has revealed that adenylate cyclase activation by toxin in a variety of tissues and species is invariably associated with ADP ribosylation of a membrane protein (M, 42,000).

We reported that the activation of adenylate cyclase in a pigeon erythrocyte lysate by cholera toxin required several soluble cofactors, namely NAD⁺ (14), GTP (15, 16), and a cytosolic protein factor with an apparent molecular weight of about 20,000 (16). These cofactors must be present at the same time as the toxin for adenylate cyclase to be activated. The NAD⁺ serves as a substrate for the toxin-catalyzed ADP ribosylation of several erythrocyte membrane proteins including the GTP-binding protein described above. The requirement for the second cofactor, GTP, was not previously understood, but in view of its regulatory role in the adenylate cyclase system it has been attractive to think that GTP might act by inducing some conformational change that enabled the GTP-binding protein to serve as a substrate for cholera toxin.

We provide here evidence that the occupation of a specific
Cofactors for Adenylate Cyclase Activation by Cholera Toxin

GTP-binding site in the erythrocyte membrane by GTP is indeed a prerequisite for the toxin-catalyzed ADP ribosylation of the GTP-binding protein (M, 42,000) and for the concomitant activation of adenylate cyclase. We show also that the binding achieved by GTP alone is in some ways limited because factors bound to the toxin, the unidentified macromolecule, either modifies the manner in which the nucleotide is bound or is itself simultaneously bound nearby.

MATERIALS AND METHODS

Cholera Toxin and Antitoxin—Cholera toxin purified by R. A. Finkelstein, the University of Texas, Southwestern Medical Center, Dallas, Texas (17) was obtained through the National Institute of Allergy and Infectious Diseases and stored at -20°C in 50% glycerol. Horse antiserum against cholera toxin (lot E1006-10A, 7700 antitoxin units/ml) was kindly provided by Dr. N. Ohtomo, Chemo-Therapentic Research Institute, Kumamoto, Japan.

Chemicals—GTP, ATP, CTP, TTP, UTP, Gpp(NH)p, App(NH)p, DNase I, micrococcal nuclease, and GTP-agarose were purchased from Sigma. GTP-Sepharose was prepared as reported by Pfeuffer (13). ([32P]ATP was a product of New England Nuclear. Horse antiserum against cholera toxin (lot E1006-10A, 7700 antitoxin units/ml) was kindly provided by Dr. N. Ohtomo, Chemo-Therapeutic Research Institute, Kumamoto, Japan.

Electrophoresis and Autoradiography—Erythrocyte ghosts treated with cholera toxin in the presence of [32P]NAD and other cofactors were washed with Buffer A, frozen, and then washed with any cells that had resealed and washed once more with the buffer. The membrane pellet was then suspended either in 50 µl of Buffer A containing 200 units/ml of beef pancreas DNase I, 1 mM MgCl₂, and 0.1 mM CaCl₂, or in 50 µl of 10 µg/ml of micrococcal nuclease in 100 mM NaCl, 2 mM MgCl₂, 1 mM CaCl₂, and 20 mM Tris, pH 8.5. After digestion of DNA for 10 min at 37°C, the membranes were washed and dissolved with heating in gel sample buffer containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described (9), and the dried gel was exposed to XR-5 x-ray film.

RESULTS

Requirement for GTP or Its Analogs in the Toxic-reaction Activation—We have reported that GTP is required for the activation of adenylate cyclase in vitro by cholera toxin (16). GTP must be present during the reaction catalyzed by the toxin, quite apart from the GTP that must be present subsequently to demonstrate the increased cyclase activity, as reported by other investigators (2-6).

The requirement for exogenous GTP is most stringent when endogenous GTP in the lysate is depleted by preincubation and when magnesium is absent from the toxin incubation. In the presence of magnesium, resident nucleoside diphosphate kinases are active and can activate ATP or other triphosphates to generate GTP from endogenous GMP or GDP (16). Therefore, we obtained the most revealing results when we washed the ghosts extensively with EDTA and excluded magnesium from the conditions prevailing during the toxin reaction, ample GTP being present during the subsequent cyclase assay. Likewise cholera toxin catalyzed the ADP ribosylation of the GTP-binding protein (M, 42,000) only in the presence of GTP or

µg/ml of pyruvate kinase, 100 µM GTP unless mentioned, and NaOH to pH 7.0. The suspension was incubated for 30 min at 37°C, and the reaction was stopped by immersion in boiling water for 90 s. To determine the amount of cyclic AMP formed, 500 µl of sodium acetate buffer, pH 5.0, containing exactly 5 pmol of cyclic [8-3H]AMP were added to the tube, insoluble material was removed by centrifugation, and the specific activity of the cyclic AMP in the supernatant was determined (19). Cyclase activities are quoted as picomoles of cyclic AMP·h⁻¹ per microcriliter of packed ghosts. One microcriliter of packed ghosts contains about 5 µg of membrane protein.

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Hemoglobin—Pigeon hemoglobin was partially purified from the erythrocyte cytosol. Cytosolic protein precipitated between 50 to 75% saturation of ammonium sulfate, pH 6.5, was diazylated against Buffer A and subjected to Sephadex G-75 chromatography in the same buffer. The eluate was monitored at 540 nm, and the hemoglobin-rich fractions were pooled. Hemoglobin precipitated again by 80% saturated ammonium sulfate, pH 6.5, followed by extensive dialysis against Buffer A, was concentrated to a final A₂₈₀ = 50 by ultrafiltration through an Amicon UM-10 membrane.

Preactivation of Erythrocyte Ghost Membranes with Gpp(NH)p—Samples of 50 µl containing 10 µl of packed ghosts (supplying 0.2 mM EDTA), 0.1 mM MgCl₂, 30 µM Gpp(NH)p, and 3 µl of the cytosol macromolecular fraction (final A₂₈₀ = 25-32) were incubated at 25°C or at 37°C for the time indicated. Control mixtures contained, in place of cytosol, the equivalent concentration of pigeon hemoglobin. This was necessary to minimize inactivation in the ghosts. Empirically, pigeon hemoglobin was more protective than ovalbumin or serum albumin. The preincubated ghosts were frozen, thawed, and then washed with 30 volumes of Buffer A containing 0.2 mM EDTA and 0.1 mM MgCl₂.

Adenylyl cyclase was then activated in this medium by incubating with cholera toxin and NAD⁺ without any added nucleoside triphosphate and with pigeon hemoglobin (A₁₆₀ = 32) replacing the cytosol macromolecular fraction. Freezing and thawing after the activation were omitted.

Results

1 The abbreviations used are: Gpp(NH)p, guanylyl-5'-yl imidodiphosphate; App(NH)p, adenylyl-5'-yl imidophosphate.
ITP (Fig. 1). With no added nucleotide, or with ATP, CTP, TTP, or UTP, the band (M, 42,000) was only faintly labeled. The need for a nucleotide in the activation of adenylate cyclase by toxin can thus be attributed to its involvement in the ADP ribosylation of the GTP-binding protein. GTP (or ITP) is likewise required for ADP-ribosylation beyond the point at which the cyclase activity is maximal, when we suppose that more ADP ribosyl protein (M, 42,000) is formed than catalytic moieties of the cyclase is available (9).

Under the conditions used the protein (M, 42,000) was always the dominant labeled product, but some other bands were ADP ribosylated to lesser extents. Nucleotides had the same proportional effect upon the labeling of them all.

The required concentration of GTP was over 100 μM at 37°C because of rapid hydrolysis but was considerably lower at cooler temperatures. At 25°C, using 25 ng/ml of cholera toxin in the presence of cytosolic macromolecular fraction (A280 = 16) and 5 mM NAD, the half-maximally effective concentration of GTP for cyclase activation lay between 3 and 10 μM, when GTP alone was added, or between 1 and 3 μM when 5 mM ATP was added too. This amount of ATP had no effect alone but served to maintain the GTP concentration by reducing hydrolysis; ITP was much less effective than GTP at these lower concentrations; about 30 μM was needed for half-maximal effect in the presence of 5 mM ATP. A similar low effective GTP concentration and the same orders of potency, GTP > ITP at low concentrations and ITP > GTP at high concentrations, have been found for the direct stimulation of avian erythrocyte adenylate cyclase by hormones (20, 21).

Thus the GTP (or ITP) probably acts at the same site in the two situations.

We found that we could replace GTP by its hydrolysis-resistant analog, Gpp(NH)p. Because Gpp(NH)p itself is a potent activator of adenylate cyclase and we wanted to distinguish the increased cyclase activity due to the toxin from that due to the nucleotide, for Fig. 2 we limited the Gpp(NH)p effect by activating with toxin and Gpp(NH)p at a low magnesium concentration (0.1 mM Mg2+ and 0.2 mM EDTA, giving about 4 μM free Mg2+). The inclusion of 100 μM GTP in the subsequent cyclase assay reduced the toxin-independent activity obtained after incubation with Gpp(NH)p, probably by promoting the displacement of bound Gpp(NH)p. Under these conditions incubation with Gpp(NH)p alone led to relatively little permanent increase in cyclase activity, and we could clearly see that the analog was as effective as GTP in supporting the toxin-catalyzed activation (Fig. 2); half-maximum activation was achieved with 1 to 3 μM Gpp(NH)p, and in this case it was not, of course, necessary to add an excess of ATP to prevent hydrolysis of guanylnucleoside triphosphate. The enhanced activation was prevented by prior addition of an antiserum containing antibodies against cholera toxin. We showed, furthermore, that the toxin-dependent [32P]ADP ribosylation was supported by low concentrations of Gpp(NH)p which was as effective as GTP with excess ATP but more effective than GTP alone. The effects were specific. The corresponding ATP analog, App(NH)p, was only about one-hundredth as effective as Gpp(NH)p in activating cyclase on its own or in supporting the toxin-dependent activation. Even these limited effects were probably attributable to a contaminant with Gpp(NH)p-like activity (perhaps inosinyl-5'-yl imidodiphosphate) present in the commercially available App(NH)p (22).

These results suggest that the binding of a guanylnucleotide to the guanylnucleotide-binding protein that regulates adenylate cyclase, presumably with allosteric consequences but without hydrolysis, is necessary for the toxin action.

**Preactivation of Erythrocyte Ghost Membranes by Gpp(NH)p in the Presence of Cytosolic Protein Factor**—Despite the conclusion just reached it is not possible to isolate a toxin-sensitive complex containing bound GTP after preincubating ghost membranes with GTP. We suppose that the

![Fig. 1. Requirement for nucleoside triphosphates during the ADP ribosylation of the GTP-binding protein by cholera toxin.](image)

Adenylate cyclase was activated by 10 ng/ml of cholera toxin in the presence of 5 μM [32P]NAD, the cytosolic macromolecular fraction (A280 = 20), and 1 mM nucleoside triphosphate. Portions of the treated ghosts were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and the ADP ribosylated proteins were detected by autoradiography. Lane 1, no toxin and no nucleotide; 2, no nucleotide; 3, GTP; 4, ATP; 5, CTP; 6, TTP; 7, UTP; 8, ITP. The major band in lane 3 represents about 500 copies/ghost of ADP-ribosyl protein (M, 42,000).

![Fig. 2. Effect of Gpp(NH)p and App(NH)p concentrations on the activation of adenylate cyclase by cholera toxin.](image)

Ghosts were preincubated for 15 min at 37°C in the presence of the cytosolic macromolecular fraction (A280 = 20) and the indicated concentration of nucleotide. The mixture contained 0.2 mM EDTA and 0.1 mM MgCl2. Cholera toxin to a final concentration of 25 ng/ml and 5 mM NAD+ were then added, and the incubation was continued for 30 min at 37°C to activate adenylate cyclase. Gpp(NH)p and cholera toxin; ○, Gpp(NH)p alone; ■, App(NH)p and cholera toxin; □, App(NH)p alone. Gpp(NH)p and cholera toxin, but 1 μl of antitoxin was added with the toxin, and the mixture was incubated for 3 min at 37°C before the addition of NAD. Empirically 1 μl of antitoxin was sufficient to neutralize the enzymic activity of 15 ng of subunit A of cholera toxin.
binding of GTP to the relevant site is readily reversed on washing or that the bound GTP is readily hydrolyzed, or both. In any case, ghosts preincubated with GTP and then washed were refractory to the toxin until additional GTP is supplied. We were able, however, to perform an equivalent experiment using Gpp(NH)p which is not only resistant to hydrolysis but forms a relatively stable complex at the nucleotide-binding site. The experimental design is shown in Table I. Pigeon erythrocyte ghosts were incubated twice and were washed between the two incubations. Cholera toxin and NAD' were present only during the second incubation. If no factors were added during the first incubation then cytosolic protein fraction and Gpp(NH)p had both to be present during the second incubation for a maximal response. Gpp(NH)p alone had relatively little effect and cytosol alone almost none. Inclusion of Gpp(NH)p in the first incubation resulted in the partial activation of the cyclase during the second incubation. Furthermore, inclusion of the cytosolic protein fraction with Gpp(NH)p in the first incubation mixture markedly enhanced the subsequent activation of the cyclase by the toxin. Providing suitable conditions were maintained it was not necessary to add either factor during the postincubation, although the additional presence of the cytosolic protein fraction during the second incubation did increase the final activity somewhat.

The preactivation of ghost membranes was dependent both on the temperature and on time, as shown in Fig. 3. At 37°C the membranes were preactivated to the maximum by 30 μM Gpp(NH)p in 15 min, while the reaction proceeded about three times more slowly at 25°C.

To see if the Gpp(NH)p and the cytosolic protein fraction required for maximum preactivation exerted their effects independently, we examined the effects of preincubating ghosts with the two components separately. In the experiments shown in Table II, ghosts were subjected to three incubations, the first with cytosolic protein fraction or Gpp(NH)p, the second with the reciprocal factor, and the third with toxin and NAD'. Gpp(NH)p alone, in either the first or the second incubation, partly supported the toxin-dependent activation of adenylate cyclase, but there was little or no further increase of the cyclase activity by including the protein fraction in the

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Adenylate cyclase activity</th>
<th>Preincubation</th>
<th>Postincubation with cholera toxin</th>
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<tr>
<td>Cytosolic fraction</td>
<td>Guanine nucleotides</td>
<td>Cytosolic fraction</td>
<td>Guanine nucleotides</td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>Gpp(NH)p</td>
<td>-</td>
<td>+</td>
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<td>Gpp(NH)p</td>
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<tr>
<td>Gpp(NH)p</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>GTP</td>
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<tr>
<td>GTP</td>
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</tbody>
</table>

*In the third incubation.

alternate incubation. The maximum preactivation was observed only when the membranes were preincubated in the simultaneous presence of Gpp(NH)p and the cytosolic protein fraction. Thus the activation of adenylate cyclase by cholera toxin can be divided into two stages, 1) the preliminary quasi-permanent binding of Gpp(NH)p (and by extension the reversible binding of GTP) to membranes in the presence of cytosolic protein factor and 2) the subsequent ADP ribosylation of a complexed guanyl nucleotide-binding protein by the toxin and NAD'.

By contrast the cytosolic protein fraction is not required for the simple activation of adenylate cyclase by GTP or Gpp(NH)p. Erythrocyte ghosts were preincubated with Gpp(NH)p, 0.1 mM magnesium, and at intervals washed ghosts were assayed for adenylate cyclase activity, without GTP. The gradual increase in cyclase activity that presumably reflects Gpp(NH)p binding was not accelerated by cytosolic proteins.

Properties of the Cytosolic Factor—The experiments in
Tables I and II also show that the effect of the cytosolic factor is durable if Gpp(NH)p is used for the preactivation. In the presence of GTP, on the other hand, the effect of the cytosolic factor is rapidly expressed and is readily reversed. This is implicit in Table I, last line, and was examined kinetically in the experiment of Fig. 4. When all the reaction ingredients were mixed, except for the cytosolic protein fraction, the toxin-dependent activation of adenylate cyclase started without measurable delay upon addition of cytosol (Fig. 4A). Conversely, the rate of activation rapidly decreased when the cytosolic factor concentration was reduced, that is when the reaction mixture was diluted into a solution of hemoglobin containing toxin, NAD*, and GTP (Fig. 4B). Under appropriate conditions the rate of toxin-dependent cyclase activation depended linearly on the concentration of cytosolic protein.

As we reported before (16) the cytosolic factor is sensitive to trypsin and presumably is, or contains, a protein. We have also found that it survives treatment with ribonuclease or micrococcal nuclease and so presumably has no polynucleotide component.

We have not been able to show that the cytosolic factor binds GTP. The factor activity in the cytosolic macromolecular fraction was not retained specifically on a commercially available GTP-agarose affinity column or on GTP-Sepharose which we prepared by Pfeuffer's method (9).

The β-adrenergic antagonist propranolol has no effect on these effects of cytosol.

Stimulation of Preactivation by Isoproterenol—Since the binding of guanyl nucleotides (2, 3, 12) and the activation of cyclase by Gpp(NH)p are known to be accelerated by catecholamines, we anticipated that isoproterenol would also increase the rate of Gpp(NH)p preactivation. We found that such an effect was demonstrated most conveniently by depleting a fresh erythrocyte lysate of GTP, and incubating it with Gpp(NH)p, with or without isoproterenol, and assaying in the presence of propranolol. Isoproterenol raised the rates of increase of cyclase activity and ability to be ADP ribosylated by about the same amount. It also shortened a lag normally observed. The similarity of the time courses (Fig. 5) lend further credence to the suggestion that Gpp(NH)p exerts both effects by binding to the same site. Evidently hormones stimulate cholera toxin's action only when guanyl nucleotide binding is the rate-limiting step. This is not the case, for example, with 1 mM GTP, in whose presence catecholamines have no effect on the rate of toxin-catalyzed ADP ribosylation.

Isoproterenol does not lift the requirement for a cytosolic protein factor in the action of cholera toxin.

**Fig. 5. Effects of isoproterenol and EDTA on the rates of activation and preactivation of adenylate cyclase by Gpp(NH)p.** Lysed erythrocytes were preincubated 90 min at 37°C to deplete endogenous GTP, chilled to 0°C, mixed with 50 µM Gpp(NH)p, distributed in 20-µl portions, and incubated at 37°C for up to 20 min alone (○), with 50 µM isoproterenol (□), or with 10 mM EDTA (△). The aliquots were frozen and thawed. Propranolol, 10 µM, was present in all subsequent steps. The ghosts were washed in 1 ml of Buffer A. For the lefthand panel adenylate cyclase activities were then measured directly without adding GTP to the assay medium. For the righthand panel an identical set of ghosts was incubated with pigeon hemoglobin (Aw = 48), 10 µg/ml of activated toxin, 10 mM thymidine, and 5 µM [32P]NAD, 25°C for 30 min, washed, digested with nuclease, washed, and analyzed by gel electrophoresis. After autoradiography the bands (M, 42,000) were excised and counted by Cerenkov radiation. Counts were corrected for the background incorporation estimated from adjacent regions of the gels. ADPR, ADP ribose.

**TABLE III**

*Modification of Gpp(NH)p binding*

<table>
<thead>
<tr>
<th>First addition</th>
<th>Second addition</th>
<th>Adenylate cyclase</th>
<th>ADP ribose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol of cyclic AMP</td>
<td>fmol/µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gpp(NH)p, 50 µM</td>
<td>0.03</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Gpp(NH)p</td>
<td>1.07</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>EDTA, 10 mM</td>
<td>0.19</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>EDTA + MgCl₂, 11 mM</td>
<td>1.17</td>
<td>6.1</td>
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<tr>
<td>NaF, 10 mM</td>
<td>0.53</td>
<td>4.3</td>
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<tr>
<td>Gpp(NH)p, 50 µM</td>
<td>0.09</td>
<td>1.1</td>
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<td>Gpp(NH)p, isoproterenol, 50 µM</td>
<td>1.30</td>
<td>10.6</td>
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<td>Gpp(NH)p, isoproterenol, GTP</td>
<td>0.87</td>
<td>3.9</td>
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</table>

**Fig. 4. Reversible action of the cytosolic macromolecular factor.** Adenylate cyclase in erythrocyte ghosts was activated at 25°C by cholera toxin (25 ng/ml) as described under "Materials and Methods." A, ghosts were incubated in 50 µl of medium containing the toxin, 5 mM NAD, 1 mM GTP, and pigeon hemoglobin (Aaw = 24) to prevent the inactivation of the cyclase. After 10 min of incubation, as indicated by the arrow, activation was started by the addition of 50 µl of cytosolic macromolecular fraction (●) or pigeon hemoglobin (○) (Aaw = 48 for both) containing the toxin, NAD*, and GTP to maintain the concentrations of these ingredients. B, the mixture containing ghosts, cholera toxin, NAD*, GTP, and the cytosolic macromolecular fraction (Aaw = 36) in 50 µl was incubated for 10 min and diluted (arrows) with 200 µl of medium composed of cholera toxin, NAD*, GTP, and either the cytosolic macromolecular fraction (●) or pigeon hemoglobin (○) (Aaw = 36 for both).
EDTA (10 mM) eliminated activation of adenylyl cyclase by Gpp(NH)p and greatly reduced its ability to preactivate for ADP ribosylation (Fig. 5). Both effects were restored by magnesium, although the amount required (4 μM) was small.

GTP reduces Gpp(NH)p binding by competing for the binding site. As expected it reduced preactivation in parallel. There was less reduction in the presence of isoproterenol. Sodium fluoride (10 mM) was found to reduce Gpp(NH)p binding and preactivation. The permanent adenylyl cyclase activation that develops when isolated membranes are incubated with sodium fluoride is not apparent in the presence of concentrated cytosol. Thus when lysed erythrocytes are incubated with Gpp(NH)p the eventual adenylyl cyclase activity is reduced, not increased, by fluoride.

**DISCUSSION**

GTP and cytosolic protein are required in addition to NAD+ for the ADP ribosylation of the GTP-binding protein (M, 42,000) by cholera toxin and the consequent activation of adenylyl cyclase. The GTP may be replaced by Gpp(NH)p or, to a lesser extent, by ITP. The effectiveness of Gpp(NH)p indicates that hydrolysis of a terminal phosphodiester bond is not necessary for the toxin-catalyzed reaction. Furthermore, we have found that the effect of the Gpp(NH)p is only slowly reversed, so that membranes can be preactivated by prior incubation with Gpp(NH)p, washed, and subsequently ADP ribosylated. Significantly, the cytosolic effect is exerted during the preactivation. Cytosolic protein is required for the successful binding of Gpp(NH)p to form the preactivation complex and must in some way modify or control the nucleotide binding. By extension we assume that the cytosolic factor is also involved in the appropriate binding of GTP for ADP ribosylation. In this case, probably because bound GTP is not necessary for the toxin-catalyzed reaction. Furthermore, the nucleotide per se.

The binding of a relatively large amount of Gpp(NH)p that is irrelevant to the preactivation has made it impossible for us to use radioactive Gpp(NH)p to directly identify the binding site during membrane preactivation, but it is clearly tempting to suppose that guanylnucleotides may assist in the toxin reaction while bound to their known binding site on the GTP-binding protein (M, 42,000). Since hydrolysis of the GTP seems unimportant, the nucleotides presumably act allosterically, altering the binding protein's conformation in such a way that it becomes a substrate for cholera toxin. The interaction of nucleotides with the binding protein is a phenomenon that has been extensively studied, indirectly, by measuring the effect of nucleotides on basal and hormone-stimulated adenylyl cyclase activities. The hypothesis that nucleotides bind to the same site for the support of ADP ribosylation and for the direct activation of cyclase is supported by the following similarities in properties. In both cases 1) the effect of GTP is expressed rapidly but is readily reversible. 2) Gpp(NH)p is effective; its actions can be slow to develop but are persistent and at least in certain circumstances the time courses are similar. 3) Similar and depend in similar ways on the temperature (Fig. 3). 4) Micromolar amounts of GTP or Gpp(NH)p, or rather more ITP, are effective while at millimolar concentrations ITP may be slightly more effective than GTP (Figs. 1 and 2). 4) The effect of Gpp(NH)p, at least, requires the presence of magnesium (Fig. 5). (EDTA reduces the activation by cholera toxin in the presence of GTP by only about 30 to 40%. It is difficult to say if magnesium is required specifically for the effect of GTP on the cyclase activity, as it must be present in the assay mixture for other reasons.) 5) Isoproterenol accelerates the Gpp(NH)p binding and reduces an initial lag (Fig. 5). 6) Development of the persistent effect of Gpp(NH)p is reduced by GTP (Table III). 7) In the presence of cytosol development of the persistent effect of Gpp(NH)p is reduced by fluoride (Table III).

The most significant difference is that a cytosolic factor is needed together with the guanylnucleotide to prepare the binding protein for ADP ribosylation but not for binding of the nucleotide per se. We suppose that the factor is a protein because the effect of cytosol is reduced by trypsin but not by nucleases. It is presumably a common constituent of cells for almost all vertebrate cells respond to cholera toxin. We have demonstrated factor activity in the cytosol of chicken, turkey, rabbit, beef, rat, human, and frog erythrocytes. We have not been able to replace cytosol by calmodulin (16), myokinase, pyruvate kinase, actin, profilin, cytochrome c, glyceraldehyde-3-phosphate dehydrogenase, tRNA, or aminoacyl-tRNA. It could be similar to the epinephrine stimulation factor that Pecker and Hanoune (24) found in the cytosol of rat liver, although cytosol is not needed for the maximal response of pigeon erythrocyte cyclase to catecholamines. On gel filtration the activity elutes at a position corresponding to that of a globular protein of about 20,000 M. (16). The rate of ADP ribosylation can vary linearly with the factor concentration.

In the absence of cytosolic factor, significant ADP ribosylation can occur but only with a 1000-fold or greater increase in toxin concentration (1). Cytosol's effect is manifest rapidly when it is added to a mixture of membranes, toxin, NAD+, and GTP (Fig. 4A, but its continuous presence is required to maintain the GTP-binding protein in a state in which it can be ADP ribosylated (Fig. 4B). The combined effect of cytosol and Gpp(NH)p, instead of GTP, is, however, sufficiently permanent that preactivated membranes can be washed free of unbound factors and subsequently ADP ribosylated. There are several models which could explain those observations. One plausible interpretation is that the cytosolic factor itself binds to one of the components of the adenylyl cyclase system in a co-operative interaction involving the guanylnucleotide. GTP hydrolysis could result in dissociation of the cytosolic factor; thus if GTP is used the continuous presence of cytosol would be necessary for cholera toxin to act. The quasi-permanent binding of Gpp(NH)p on the other hand would produce a quasi-permanent binding of the cytosolic factor in a complex having sufficient stability to be identified. It is also possible to construct hypotheses in which the cytosolic factor alters the interaction between guanylnucleotides and the binding protein without remaining bound itself.

If it is true that the Gpp(NH)p which binds to the regulatory protein in the presence of cytosol can be detected equally by assaying cyclase activity or by measuring the number of ADP-ribosylatable sites, we can directly determine the catalytic activity per functional Gpp(NH)p site which, at least when only a few Gpp(NH)p are bound, may be the same as the catalytic activity per functional catalytic moiety. In Fig. 5 and similar experiments where such comparisons seem valid, we have found ratios in the range of 160 to 312 cyclic AMP molecules per min per ADP ribose residue.

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Cofactors for Adenylate Cyclase Activation by Cholera Toxin

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