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Requirement for Guanosine Triphosphate in the Activation of Adenylate Cyclase by Cholera Toxin

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The activation of adenylate cyclase in lysed pigeon erythrocytes requires, among several cofactors, a nucleotide which may be ATP, GTP, or many other triphosphates. However, after removal of endogenous nucleotides by gel filtration or by adsorption onto charcoal the requirement can be met only by GTP, or an analog of GTP. The GTP is required during the activation of the cyclase by toxin even if GTP is also included during the subsequent adenylate cyclase assay, conducted without toxin. In the presence of GTP it is possible to assay for the cytosolic protein that is also required for the action of cholera toxin. By gel filtration, its apparent molecular weight is 15,000–20,000.

Key words: cholera toxin, GTP, pigeon erythrocyte, adenylate cyclase, cytosolic factor, phosphodiesterase protein activator

Fragment A_1 of cholera enterotoxin catalyses the activation of adenylate cyclase (ATP pyrophosphate-lyase [cyclizing] E.C. 4.6.1.1.) in broken cells [1]. Besides plasma membranes, as a source of the cyclase, there are several soluble factors, omission of any one of which reduces the ability of the system to respond to the toxin and thus increases the toxin concentration required for a given response. We and others have attempted to identify the relevant factors and hitherto have recognized four necessary components of the cytosol, namely NAD, nucleoside triphosphate, an unidentified protein, and thiol compounds that can reduce the cystine in toxin's subunit A [2]. The NAD requirement is specific, for it acts as an ADP-ribosyl donor [3, 4]. The nucleoside triphosphate requirement is less specific and can be met by ATP, CTP, UTP, GTP, or even by their imido or

ATP, CTP, GTP, TTP, UTP: adenosine, cytosine, guanosine, thymidine, and uridine triphosphate; ADP, GDP: adenosine, guanosine diphosphate; AMP, GMP: adenosine, guanosine monophosphate; NAD: nicotinamide adenine dinucleotide.

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methylene analogs such as App(NH)p, but not by ADP, AMP, cyclic AMP, ADP-ribose, and so on.

In the presence of saturating amounts of NAD and ATP, and using prereduced toxin, the erythrocyte cytosol is still required for maximum sensitivity. It provides a necessary protein but efforts to purify this protein have been generally unsuccessful. Even though the cytosol is fairly stable in the cold, dialysis, gel filtration, ion exchange chromatography, and such fractionation techniques incur significant losses of activity. We have now found that a reason for this inactivation is the removal of guanine nucleotides and that GTP must be present for cyclase activation by cholera toxin.

MATERIALS AND METHODS

Concentrated pigeon erythrocyte lysates were separated by centrifugation into ghost and cytosol fractions. The ghosts were washed three times with about 10 volumes of buffer (0.13 M NaCl, 0.01% NaN_3, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.3). All columns were equilibrated in the same buffer. The cytosol was applied to a Sephadex G25 column and the effluent was monitored at the hemoglobin absorption maximum, 540 nm. The input had an OD_{540} of 80–100 and the pooled macromolecular fraction about 40–50. Adenylate cyclase was activated in a volume of 50 μl containing 10 μl of packed ghosts, 5 mM NAD, nucleoside triphosphates, and 30 μl cytosol or macromolecular fraction as indicated. Activation, for 10 min at 37°C, was started by the addition of 200 ng/ml of cholera toxin which had been incubated with 0.5% sodium dodecyl sulfate (SDS) and 5 mM dithiothreitol to liberate fragment A_1 [2]. Except for Figs. 1, 2, 5, 6 and Tables VI and VII, the mixture was then frozen to lyse any resealed ghosts. Freezing results in a substantial reduction of adenylate cyclase activities but was considered desirable to avoid high concentrations of nucleotides and of toxin in a subclass of ghosts during the subsequent cyclase assay. The ghosts were diluted in 30 volumes of buffer, collected by centrifugation, and assayed for adenylate cyclase activity as reported [2].

RESULTS

Preincubation of total cytosol results in the hydrolysis of its store of ATP and the emergence of an "ATP" requirement [2]. Hydrolysis is more rapid at 37° than at 20° (Fig. 1). No other damage is detectable and the cytosol is restored by ATP to about its original ability to the toxin activation of the cyclase.

Dialysis, even against ATP, soon results in almost complete loss of activity of the retentate. We were not able to restore activity by adding back reconcentrated diffusate. However, we were able to maintain activity during dialysis by immersing the bag in a concentrate of small molecules prepared separately by gel filtration on Sephadex G25. This suggested that the small factor was not ATP.

Other separation methods lead to the same conclusion. Passage of cytosol over Sephadex G25 causes a much greater loss of activity than can be accounted for by dilution (Fig. 2). In Table I we compare the macromolecular fraction prepared on G25 with the original cytosol diluted to the same protein concentration. There is little cyclase activation in either case without a nucleoside triphosphate, but whereas ATP is partially effective with the diluted cytosol, it is ineffective with the G25 fraction.

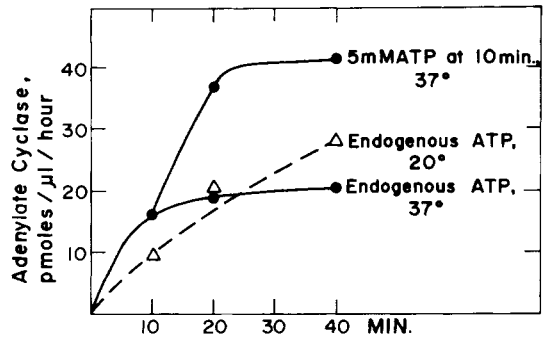

Fig. 1. Progress of adenylate cyclase activation in an erythrocyte lysate incubated with 0.1 mM NAD, 100 ng/ml activated cholera toxin and with extra ATP only where indicated.

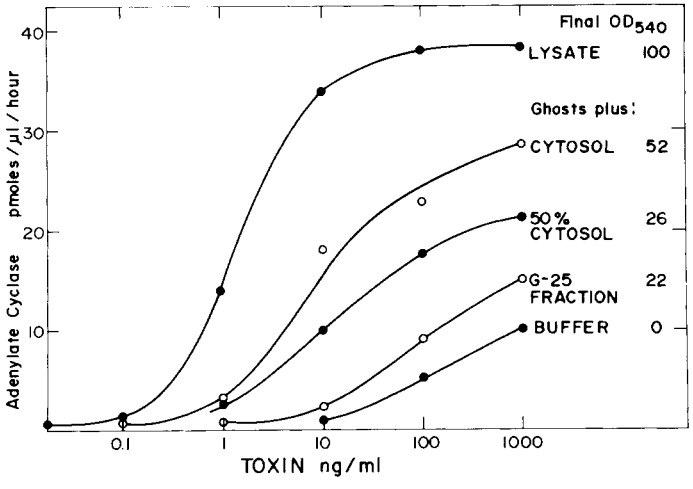

Fig. 2. Representative toxin concentration-response curves for adenylate cyclase activation in an unfractionated pigeon erythrocyte lysate and in lysates reconstituted from washed ghosts and cytosol, cytosol diluted in buffer, the macromolecular peak obtained by fractionation on G25, or buffer only (5 mM ATP, 5 mM NAD, 37°, 30 min).

TABLE I. Comparison of Whole Cytosol and the Macromolecular Fraction Prepared on G25 in the Activation of Adenylate Cyclase (activity, pmoles cyclic AMP/μl ghosts/h)

Nucleoside triphosphates	Source of macromolecules		
	Cytosol	G25 fraction	None
None	6.3	6.4	3.0
1 mM ATP	8.6	6.1	1.8
5 mM ATP	11.2	5.3	2.9
1 mM GTP	19.0	14.5	3.2
5 mM GTP	19.6	16.9	3.3
5 mM GTP + 2 mM ATP	19.9	19.6	2.9

Fresh cytosol was diluted to the same protein concentration as the G25 fraction. In both cases the OD_{540} of the incubated mixture was 33.

On the other hand we found that GTP greatly stimulates the cyclase activation both with diluted cytosol and with the G25 fraction. ATP added with GTP often enhances the activation somewhat but GTP has the greater effect. CTP, UTP, TTP, and cyclic GMP were also ineffective (Table II). A pigeon erythrocyte lysate contains GDP and GMP and the phosphotransferases to convert them to GTP in the presence of ATP or another nucleoside triphosphate. Thus the addition of ATP alone may suffice for maximum activation using whole cytosol. However, after removal of small molecules by gel filtration it is necessary to add GTP itself, or a GTP-generating system consisting of GDP or GMP and ATP. As expected, in this case App(NH)p, a hydrolysis-resistant analog of ATP, is ineffective (Table III).

We have obtained analogous results by adsorbing endogenous nucleotides from cytosol onto activated charcoal (Table IV).

Since GTP sometimes increases adenylate cyclase activity by itself, and since even careful washing cannot eliminate the possibility that some GTP is carried over from the activation incubation into the subsequent cyclase assay, it was important to show that the nucleotide was really required for the activation by cholera toxin and not simply for the

TABLE II. Specificity of the GTP Requirement for the Activation of Adenylate Cyclase in the Presence of the Macromolecular Fraction of Cytosol Prepared on Sephadex G25

Nucleotide added	Adenylate cyclase activity (pmoles cAMP/μl/ghosts/h)
None	2.5
1 mM GTP	15.7
1 mM CTP	2.1
1 mM UTP	3.1
1 mM TTP	3.3
1 mM cyclic GMP	4.4

Final OD_{540}=28.

TABLE III. Effects of GDP and GMP on the Activation of Adenylate Cyclase in the Presence of the G25 Macromolecular Fraction of Cytosol

Nucleotides (2 mM each)	Adenylate cyclase activity (pmoles cAMP/μl ghosts/h)
None	5.7
GTP	16.8
GDP	6.1
GMP	4.2
ATP	4.9
GTP + ATP	20.1
GDP + ATP	13.0
GMP + ATP	12.1
App(NH)p	7.2
GTP + App(NH)p	14.7
GDP + App(NH)p	3.8
GMP + App(NH)p	5.2
App(NH)p (no toxin)	4.4
App(NH)p (no G25 fraction)	4.7

Final OD_{540} =24.

TABLE IV. Nucleotide Requirements Before and After Charcoal Treatment (adenylate cyclase activity, pmoles cAMP/μl ghosts/h)

	None	5 mM ATP	5 mM GTP
Buffer	2.2	2.2	2.4
Control cytosol	3.1	18.4	21.3
Charcoal-treated cytosol	1.8	2.6	24.3

Cytosol was agitated for 3 min with 30 mg/ml prewashed activated charcoal (Norit A). The charcoal was removed by centrifugation. The final OD_{540} during the activation was 43.

cyclase activity. That this was indeed the case is demonstrated in Table V. The possibility that the results might reflect the activities of a subclass of resealed ghosts was eliminated by freeze-thawing between the two assays. In addition, although the absence of NAD and cytosol is probably sufficient, antitoxin was added for the second incubation to make doubly sure that there was no further action of the toxin. The results show that GTP is definitely required during the first incubation with toxin and the macromolecular fraction of cytosol: It has no effect in the absence of either of the other factors and has little effect if added solely for the second incubation, although after intoxication there was an enhanced sensitivity to GTP, as previously reported [5]. The GTP requirement for activation is expressed rapidly. A reconstituted lysate, lacking only nucleoside triphosphate, was incubated at 37°. Activation started immediately upon, or very shortly after, the addition of 2 mM GTP (Fig. 3). Similar experiments, in which other ingredients were added last, confirm that cyclase activation requires the simultaneous presence of GTP, toxin, cytosolic macromolecules, and NAD.

TABLE V. Requirement for GTP During Activation With Toxin

First incubation (activation with toxin)		Second incubation (adenylate cyclase assay without toxin)	
1 mM GTP	G25 fraction	GTP	Adenylate cyclase activity, (pmoles cAMP/μl ghosts/h)
–	–	0	3.1
+	–	0	3.9
–	+	0	6.9
+	+	0	16.3
+	+	200 μM	22.2
–	+	200 μM	6.9
–	+	1 mM	7.7
+	+ (no toxin)	0	1.8

In the first incubation, ghosts, toxin, and NAD were incubated with GTP and the G25 macromolecular fraction (final OD_{540} = 24) as indicated. After freezing and thawing, the ghosts were washed and incubated in the adenylate cyclase assay medium containing GTP as indicated and sufficient antitoxin to inhibit 10 ng of toxin.

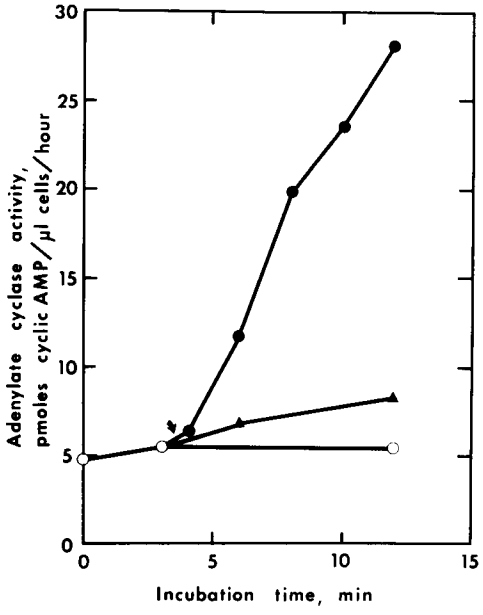

Fig. 3. Activation started by GTP. A lysate was reconstituted from washed ghosts and G25 macromolecular fraction of cytosol (final OD_{540}=29) and incubated at 37° with NAD and toxin. ○) no nucleoside triphosphate; ▲) 5 mM ATP added (arrow); ●) 2 mM GTP added (arrow).

At 37° a relatively high concentration of GTP appears necessary: The effect is significant at 0.1 mM and fairly complete at 1 mM (Fig. 4). However, as little as 3 μM GTP or Gpp(NH)p has a significant effect at 25°, at which temperature there is much less non-specific GTP hydrolysis.

Support of Toxin's Activity by Gpp(NH)p

Gpp(NH)p, an analog that has similar but greater effects than GTP on basal and hormone-stimulated adenylate cyclase activities, is also able to replace GTP in the activation by cholera toxin. The experiment (Fig. 5) was designed to distinguish the time-dependent cyclase activation by toxin in the presence of Gpp(NH)p from the very large time-dependent cyclase activation that can be caused by Gpp(NH)p itself. The effect of the toxin was maximized by reducing endogenous nucleoside triphosphates merely by preincubation, thus allowing us to use a complete, rather than a reconstituted, lysate. The relative rate of activation by Gpp(NH)p itself was reduced by incubating at 20° rather than 37° (since the Q_{10} for the Gpp(NH)p effect is larger than the Q_{10} for the toxin reaction).

The Macromolecular Cytosolic Factor

The macromolecular fraction of cytosol is required for the action of toxin, even at high concentrations of GTP or with any combination of nucleotides tested (see Tables I and V and Fig. 4). The active ingredient within this fraction is probably a protein, for the activity is sensitive to trypsin (original observation by Dale Yelton, see Table VI). Figure 6 shows the fractionation of cytosol on Sephadex G75. The ability to support the action of cholera toxin in vitro is clearly separated from hemoglobin and has an apparent size of about 15,000–20,000 daltons. The activity was retained by an Amicon UM-10 ultrafiltration membrane which has a nominal cutoff of 10,000 daltons. Purification of this protein should now be feasible.

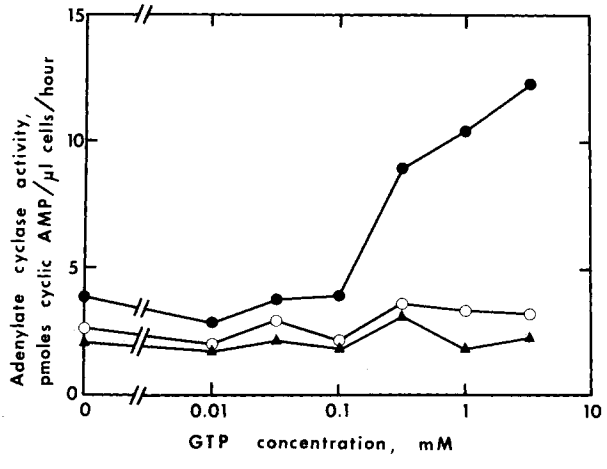

Fig. 4. Effect of GTP concentration during activation of a lysate reconstituted from washed ghosts and G25 macromolecular fraction of cytosol (final OD_{540}=29). Antitoxin and 0.2 mM GTP were present during the subsequent adenylate cyclase assay. ▲) no toxin; ○) no G25 fraction; ●) complete.

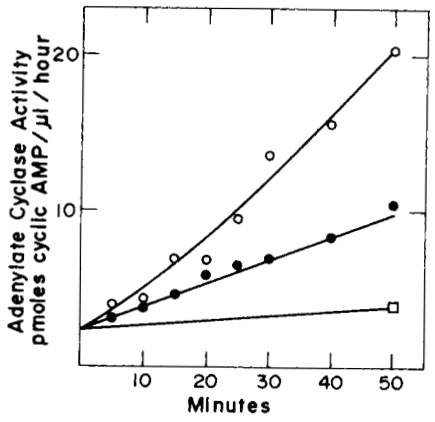

Fig. 5. Support of activation by Gpp(NH)p. The lysate was preincubated for 1h at 37° to deplete nucleoside triphosphates and then incubated at 20° with 5 mM NAD and 100 ng/ml activated cholera toxin (□), 1 mM Gpp(NH)p (●), or both toxin and Gpp(NH)p (○).

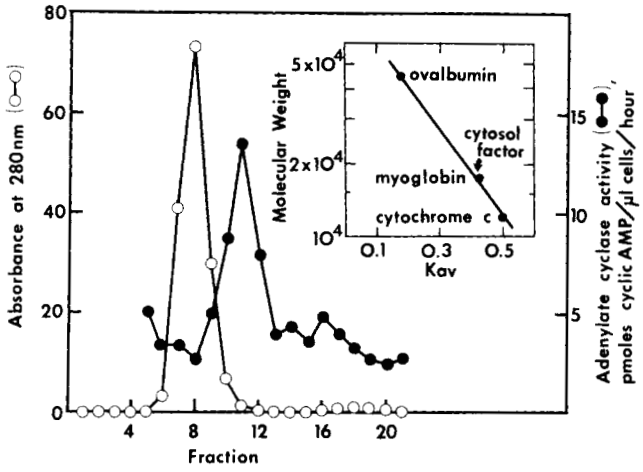

Fig. 6. Partial purification of the macromolecular cytosolic factor by gel filtration. Erythrocyte cytosol (1.5 ml) was applied to a G75 column (0.9 cm × 56 cm); 2.2 ml fractions were assayed for their ability to support cyclase activation in a mixture of washed ghosts, 5 mM NAD, 1 mM GTP, and 200 ng/ml toxin. Adenylate cyclase was assayed in the presence of 100 μM GTP.

Moss and Vaughn [6] reported that the toxin activation of solubilized brain adenylate cyclase was supported by boiled brain supernatant. The boiled supernatant could be replaced, in part, by the protein activator of cyclic nucleotide phosphodiesterase, plus calcium, and GTP. However, in our system protein activator from porcine brain (kindly provided by Claude Klee) with or without calcium is unable to replace the macromolecular cytosolic factor and is not stimulatory when present in addition to cytosol. There is no indication that endogenous protein activator might be involved, for the activation is not affected by calcium chelation (Table VII). Thus the pigeon cytosolic factor seems to be a different protein from protein activator.

TABLE VI. Inactivation of the Macromolecular Cytosol Factor by Trypsin

	Adenylate cyclase activity (pmoles cAMP/μl ghosts/h)		
a. Control cytosol	24.0	33.0	43.9
b. Cytosol preincubated with trypsin	5.2	15.0	15.5
c. Cytosol preincubated alone	21.3	26.5	32.3
d. No cytosol	1.8	2.2	5.0

Cytosol ($OD_{540} \simeq 100$) was preincubated at 37° for 10 min with 300 μg/ml of trypsin (b) or without trypsin (c). Then soybean trypsin inhibitor (final 600 μg/ml) (b) or inhibitor plus trypsin (c) was added and the incubation was continued for 3 min. Cyclase was activated with 200 ng/ml toxin, 5 mM NAD, 1 mM GTP, and cytosol to a final OD_{540} of about 70. The results of three separate experiments are shown.

TABLE VII. Lack of Effects of the Protein Activator of Cyclic Nucleotide Phosphodiesterase in the Activation of Adenylate Cyclase

	Additions for incubation with toxin		Adenylate cyclase activity
Macromolecular fraction	100 μM Ca^{2+}	1 mM EGTA	(pmoles cAMP/μl ghosts/h)
None	–	–	4.3
None	+	–	3.7
Protein activator (147 μg/ml)	–	–	4.3
Protein activator (147 μg/ml)	+	–	4.2
Protein activator (343 μg/ml)	+	–	3.1
Protein activator (147 μg/ml)	–	+	4.3
Cytosol (final $OD_{540} = 40$)	–	–	38.7 ± 2.3
Cytosol (final $OD_{540} = 40$)	+	–	31.5 ± 1.3
Cytosol (final $OD_{540} = 40$)	–	+	34.4 ± 2.1
Cytosol + protein activator (147 μg/ml)	–	–	24.3 ± 3.3
Cytosol + protein activator (147 μg/ml)	+	–	29.9 ± 3.7

Cyclase activation was conducted in 5 mM NAD, 1 mM GTP, and 200 ng/ml of toxin. Adenylate cyclase was assayed in the presence of 50 μM $CaCl_2$, whenever calcium had been present during the activation, and of 100 μM GTP in all cases. For the bottom six rows cyclase activities were determined in triplicate.

DISCUSSION

We have shown that GTP satisfies the nucleoside triphosphate requirement of cholera toxin in vitro and that the previously observed ability of ATP, UTP, or CTP to support this action was due at least in part to their ability to generate GTP from GDP or GMP in the presence of appropriate enzymes. They are ineffective once GDP or GMP is removed by dialysis, gel filtration, or charcoal adsorption. App(NH)p is ineffective in the partially purified system, even in the presence of GDP or GMP. It appears to support the intoxication in whole lysate, an effect that can reasonably be attributed to its inhibition of nucleoside triphosphatases, thus allowing endogenous GTP to survive longer [10].

The involvement of GTP in the regulation of hormone-stimulated adenylate cyclase activity has been extensively documented [7, 8]. It binds to one, or perhaps two [9], separable subunits of the adenylate cyclase complex. Recently Cassel and Selinger [10] and Levinson and Blume [11] presented evidence that cholera toxin inhibits the hydrolysis of GTP at a regulatory site and thus traps adenylate cyclase in an active (GTP-bound) form. Furthermore, we have presented evidence that the inhibition of GTPase may be due to the toxin-catalyzed addition of a residue of ADP-ribose to a 42,000-Mr GTP-binding protein. The nucleotide requirement for the ADP-ribosylation is the same as that reported here for cyclase activation [4]. A reasonable possibility is that the necessary GTP may act by binding to the target protein and altering its conformation in some manner. Indeed the amount of guanyl nucleotide effective in the toxin reaction, when measured at a low temperature to restrict nucleoside triphosphatase action, is not much greater than the amount required to modulate the hormonal stimulation of cyclase activity in isolated avian erythrocyte membranes [12, 13].

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