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Mechanism of adenylate cyclase activation by cholera toxin: Inhibition of GTP hydrolysis at the regulatory site

(3′:5′-cyclic AMP/guanyl nucleotide site/catecholamine-stimulated guanosinetriphosphatase/hormone receptor)

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ABSTRACT **Treatment of turkey erthrocyte membranes with cholera toxin caused an enhancement of the basal and catecholamine-stimulated adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activities. Both of these activities required the presence of GTP. The toxin effect on the adenylate cyclase activity coincided with an inhibition of the catecholamine-stimulated guanosinetriphosphatase activity. Inhibition of the guanosinetriphosphatase, as well as enhancement of the adenylate cyclase activity, showed the same dependence on cholera toxin concentrations, and the effect of the toxin on both activities was dependent on the presence of NAD.**

It is proposed that continuous GTP hydrolysis at the regulatory guanyl nucleotide site is an essential turn-off mechanism, terminating activation of the adenylate cyclase. Cholera toxin inhibits the turn-off guanosinetriphosphatase reaction and thereby causes activation of the adenylate cyclase. According to this mechanism GTP should activate the toxin-treated preparation of adenylate cyclase, as does the hydrolysis-resistant analog guanosine 5′-(β,γ-imino)triphosphate [Gpp(NH)p]. Indeed, the toxin-treated adenylate cyclase was maximally activated, in the presence of isoproterenol, by either GTP or Gpp(NH)p, while adenylate cyclase not treated with toxin was stimulated by hormone plus GTP to only one-fifth of the activity achieved with hormone plus Gpp(NH)p. Furthermore, the toxin-treated adenylate cyclase activated by isoproterenol plus GTP remained active for an extended period (half-time of 3 min) upon subsequent addition of the β-adrenergic blocker, propranolol. The native enzyme, however, was refractory to propranolol only if activated by Gpp(NH)p but not by GTP.

Cholera toxin has been shown to produce its pathological effects via stimulation of the intestinal adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], thus causing an increase in the level of 3′:5′-cyclic AMP in intestinal epithelial cells (1–3). Several experiments, *in vitro*, however, have shown that the effect of the toxin is not confined to the intestinal cells. In all of the hormone-responsive adenylate cyclase systems examined to date, the toxin was able to mimic the specific response elicited by cyclic AMP (4). Recently, Gill has shown that the peptide A_1 of the toxin activates the adenylate cyclase in membrane preparations of pigeon erythrocytes in the presence of NAD, cell cytosol, and ATP (5, 6). The finding that the toxin activates the adenylate cyclase in a great number of different cells suggests that it acts on a component commonly present in eukaryotic adenylate cyclase systems; however, this component has not as yet been identified.

In recent years it has been demonstrated that hormone stimulation of the adenylate cyclase is dependent on GTP binding at a regulatory site (7, 8). Substitution of GTP by the hydrolysis-resistant analog guanosine 5′-(β,γ-imino)triphosphate [Gpp(NH)p] resulted in a maximal and persistent activation of the adenylate cyclase (see ref. 9 for recent review). It was thought that the differences between GTP and Gpp(NH)p as activators of the adenylate cyclase was due to the analog's resistance to hydrolysis (10, 11). In accordance with this mechanism, we have found a catecholamine-stimulated guanosinetriphosphatase (GTPase) activity in turkey erythrocyte membrane (12). The properties of the GTPase suggested that this activity takes place at the guanyl nucleotide regulatory site. This led us to propose that hydrolysis of GTP is a turn-off mechanism that returns the activated adenylate cyclase to the basal inactive state (12, 13). The present study was aimed at evaluating the effect of cholera toxin on the GTPase reaction. The results have shown that the toxin concurrently inhibits the GTPase activity and enhances the GTP-dependent adenylate cyclase activity. Furthermore, the effects of GTP on the adenylate cyclase in preparations treated with cholera toxin resembled those seen with the hydrolysis-resistant analog Gpp(NH)p in untreated preparations. We therefore propose that cholera toxin activates the adenylate cyclase via an inhibition of the turn-off GTPase reaction.

MATERIALS AND METHODS

[α-^{32}P]ATP was obtained from the Radiochemical Centre, Amersham, England. Adenosine 5′-(β,γ-imino)triphosphate [App(NH)p] and Gpp(NH)p were from ICN. Cholera toxin was a gift of J. D. Gardner, National Institutes of Health, USA. [γ-^{32}P]GTP was prepared by the method of Glynn and Chappell (14).

Turkey Erythrocyte Membranes. These were prepared by digestion of the nuclei with DNase, as described for frog erythrocytes (15), with the following modifications: the pH of the Tris buffer used for lysis and for washes of the membranes was 7.9 at 23° and 2-mercaptoethanol (2 mM) was used instead of dithiothreitol. The membranes were stored in liquid nitrogen and thawed on the day of the experiment.

Toxin Treatment. Adenylate cyclase in membrane preparations from turkey erthrocytes was activated with cholera toxin (120 μg/ml) that had been incubated for 15 min at 37° in turkey erythrocyte cytosol containing 0.6 mM dithiothreitol.† The cytosol was prepared from packed erythrocytes lysed by freezing in liquid nitrogen, followed by thawing at 37°, sus-

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Abbreviations: Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate; App(NH)p, adenosine 5′-(β,γ-imino)triphosphate; GTPase, guanosinetriphosphatase; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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† Activation of adenylate cyclase by treatment of turkey erythrocyte membranes with cholera toxin, as described in this paper, was developed by L. H. Graff and M. Field of the Thorndike Laboratory, Harvard Medical School. We are grateful to them for generously providing us with details of their unpublished experiments.

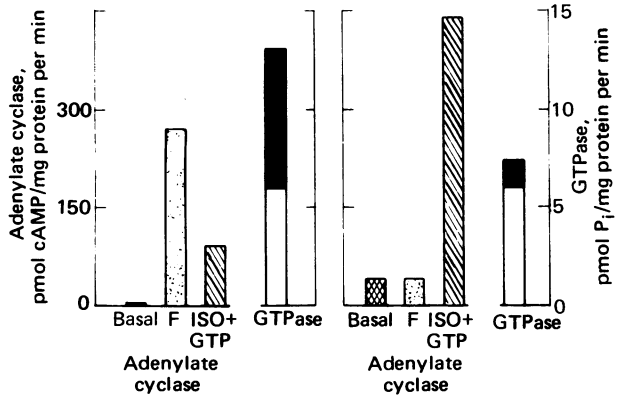

FIG. 1. Effect of cholera toxin on adenylate cyclase and GTPase activities. Turkey erythrocyte membranes were pretreated with toxin (12 μg/ml) and then washed as described in *Materials and Methods*. Adenylate cyclase was assayed in reaction mixture I, with 10 mM NaF (F) or 50 μM of both isoproterenol (ISO) and GTP. GTPase activity was determined as described in *Materials and Methods*. □, Basal; ■, catecholamine-stimulated. (*Left*) No toxin. (*Right*) Toxin.

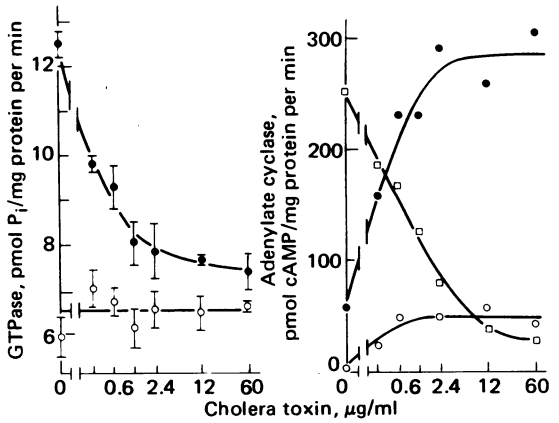

FIG. 2. Dose response curves for toxin effect on the GTPase (*Left*) and adenylate cyclase (*Right*) activities. Membranes were pretreated with the indicated concentrations of toxin. Adenylate cyclase was assayed in reaction mixture I, which contained GTP as a contaminant of the ATP substrate (see *Materials and Methods*). The concentration of NaF was 10 mM and isoproterenol was 50 μM. The results of GTP hydrolysis are the means ± SD ($n = 3$). ●, Isoproterenol; □, NaF; ○, no addition (*Left*) or basal value (*Right*).

pension in 1.5 volumes of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)/saline buffer (20 mM Hepes, pH 7.5/130 mM NaCl/5 mM KCl/2 mM $MgCl_2$/0.6 mM dithiothreitol), and centrifugation (10,000 × *g* for 10 min) to remove membranes and nuclei. Turkey erthrocyte membranes (1 mg/ml) suspended in Hepes/saline buffer were mixed with an equal volume of preincubated toxin diluted in cytosol to give the desired final concentrations. Control systems received cytosol without toxin. Other additions in final concentrations were: 1 mM NAD, 1 mM ATP, 0.6 mM dithiothreitol, 10 mM phospho*enol*pyruvate (potassium salt), 120 μg of pyruvate kinase per ml, and 20 μg of myokinase per ml. After 5 min of incubation at 37°, the reaction was terminated by addition of 20 volumes of chilled hypotonic medium (10 mM Tris·HCl buffer, pH 8.1/0.2% bovine serum albumin/2 mM 2-mercaptoethanol). The membranes were subjected to centrifugal washes with cold hypotonic medium until the supernatant was essentially free of hemoglobin (usually two or three washes). To remove the bovine serum albumin the final pellet was washed once with 10 mM Tris·HCl, pH 7.5, containing 2 mM 2-mercaptoethanol.

GTPase Assay. GTPase activity was measured as described (12), except that the concentration of App(NH)p in the assay was increased from 0.2 to 0.5 mM. This reduced the nonspecific GTP hydrolysis, which became about 10% of the basal GTPase activity. Basal GTPase activity was determined as the decrease in $^{32}P_i$ liberation due to an isotope dilution caused by the addition of 3 nmol of unlabeled GTP to the GTPase assay. Catecholamine-stimulated GTPase activity was calculated as the increase in GTP hydrolysis caused by an addition of 50 μM *d,l*-isoproterenol to the assay. Assays were performed in triplicate.

Adenylate Cyclase Assay. Activity was assayed according to Salomon *et al.* (16). Membranes (20–40 μg) were incubated for 10 min at 37° in 0.1 ml of one of the following two reaction mixtures: Reaction mixture I consisted of [α-^{32}P]ATP (1 to 3 × 10^6 cpm), 0.3 mM ATP (Sigma no. A-3377), 6 mM $MgCl_2$, 1 mM cyclic AMP, 12 mM creatine phosphate, 3 units of creatine phosphokinase, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 2 mM 2-mercaptoethanol, and 50 mM Tris·HCl buffer, pH 7.5. The ATP preparation in reaction mixture I was apparently contaminated with GTP, as can be judged by the small increase (10—30%) in adenylate cyclase activity caused by the addition of GTP. Reaction mixture II utilized a purified ATP, substantially freed from GTP contaminations (Sigma no. A-2383). The latter was used in order to determine effects of GTP on adenylate cyclase activity. The regeneration system consisted of 5 mM phospho*enol*pyruvate (potassium salt), 50 μg of pyruvate kinase per ml, and 10 μg of myokinase per ml, and the buffer was imidazole–HCl (50 mM, pH 6.7). Due to the lower pH of the assay medium, adenylate cyclase activity in reaction mixture II was about 20% lower than in reaction mixture I. Protein was determined by the method of Lowry *et al.* (17), with bovine serum albumin as standard.

RESULTS

Treatment of turkey erythrocyte membranes with cholera toxin in the presence of erythrocyte cytosol, NAD, dithiothreitol, and ATP dramatically changed the profile of the adenylate cyclase activity (Fig. 1). The previously insignificant basal activity was increased about 10-fold and the response to the β-adrenergic agonist isoproterenol plus GTP was increased 5 times in toxin-treated membranes. On the other hand, fluoride activity was completely inhibited, as fluoride did not cause any further activation above the toxin-induced basal activity. These results confirm previous findings by Field (18).

We have recently reported (12) that turkey erythrocyte membranes reveal a catecholamine-stimulated GTPase activity. It was suggested that this GTPase, which has both high affinity and specificity for GTP, regulates the hormone-responsive adenylate cyclase activity. The membranes also revealed a basal GTPase activity operating in the absence of a catecholamine hormone which is apparently not coupled to the adenylate cyclase system (12). As shown in Fig. 1, toxin treatment selectively inhibited about 80% of the catecholamine-stimulated GTPase activity while it had no effect on the basal GTPase activity. Increasing concentrations of the toxin caused progressive inhibition of the catecholamine-stimulated GTPase activity and a parallel increase in the basal and the isoproterenol-stimulated adenylate cyclase activities. Inhibition of the fluoride-stimulated adenylate cyclase activity, however, seemed to require higher doses of toxin (Fig. 2). As shown in Table 1, both toxin and NAD were required in order to produce enhancement of the basal and the isoproterenol-stimulated adenylate cyclase activity and to inhibit the stimulation by fluoride. The same requirements were found for inhibition of the cate-

Table 1. NAD dependence of cholera toxin effects on the adenylate cyclase and GTPase activities

Treatment	Catecholamine-stimulated GTPase, pmol $^{32}P_i$/ mg protein per min	Adenylate cyclase, pmol cAMP/mg protein per min		
		Basal	Isoproterenol	F^-
None	6.5	3	85	297
With:				
Cytosol	6.6	3	83	280
Cytosol + toxin	6.3	3	79	257
Cytosol + NAD	5.0	3	72	302
Cytosol + toxin + NAD	1.3	38	333	86

Membranes were pretreated for 5 min at 37° with turkey erythrocyte cytosol containing the additions described in *Materials and Methods* except that toxin (6 μg/ml) and NAD (1 mM) were added only where indicated. Erythrocyte cytosol was prepared from a cell lysate which was first incubated for 6 min at 37° to deplete endogenous NAD (5). Adenylate cyclase was assayed in reaction mixture I. The hydrolysis of GTP in the absence of isoproterenol was very similar in the various systems (6.3–6.7 pmol/mg of protein per min). Standard deviation in triplicate determinations of GTP hydrolysis ranged between ± 0.1 and 0.3 pmol/mg of protein per min.

cholamine-stimulated GTPase activity. The GTPase and adenylate cyclase activities were compared at various GTP concentrations by determining adenylate cyclase activity at the pH used for the GTPase assay (pH 6.7). The hormone-stimulated adenylate cyclase and the GTPase in a control membrane preparation showed the same high affinity for GTP (K_m of 0.1 μM, Fig. 3). Toxin treatment did not significantly change the affinity for GTP in the adenylate cyclase reaction (K_m of 0.25 μM). However, the V_{max} of the GTP plus isoproterenol-stimulated adenylate cyclase was markedly increased in the toxin-treated preparation (Fig. 3 *right*). Similar results were previously reported by Bennett and coworkers (19–21). Toxin modification of the catecholamine-stimulated GTPase decreased by 80% the V_{max} of the GTPase reaction (Fig. 3 *left*). Regardless of whether the enzyme was assayed at an optimal pH of 6.7 or at pH 7.5, the toxin inhibited the GTPase to the same extent.

If cholera toxin activates adenylate cyclase through inhibition of the GTPase, then after toxin treatment GTP should act like the hydrolysis-resistant analog Gpp(NH)p and cause maximal and persistent activation of the adenylate cyclase. Table 2 shows that isoproterenol and GTP synergistically activated the toxin-treated adenylate cyclase to the same extent as that caused by isoproterenol and Gpp(NH)p in an enzyme not treated with the toxin. The activity obtained with isoproterenol plus Gpp(NH)p was somewhat lower in the toxin-treated than in the untreated enzyme. After toxin treatment, basal adenylate cyclase activity was low in the absence of guanyl nucleotide. This basal activity was increased by GTP and by Gpp(NH)p 4 and 6 times, respectively. In preparations not treated with the toxin, Gpp(NH)p caused some increase in basal activity, whereas GTP had no effect (Table 2). A further similarity in the effects of GTP and Gpp(NH)p on the toxin-treated adenylate cyclase was the resistance of the activated enzyme to subsequent inhibition by propranolol. For the first minute after the addition of propranolol the rates of cyclic AMP production by an enzyme preactivated by isoproterenol plus either Gpp(NH)p or GTP were equal in toxin-treated membranes. On the other hand, subsequent addition of propranolol completely inhibited the adenylate cyclase preactivated by hormone plus GTP in a preparation not treated with the toxin. The resistance to propranolol of the toxin-treated adenylate cyclase, preactivated by isoproterenol plus GTP, was transient, with a half-life of about 3 min (Fig. 4). This might be explained by the finding that even at very high doses, the toxin did not cause complete inhibition of the catecholamine-stimulated GTPase (Fig. 2). There was no decay of activity in systems that did not receive propranolol or in those systems that received propranolol but had been preactivated by isoproterenol plus Gpp(NH)p (Fig. 4).

To test whether cholera toxin has an effect on the rate of adenylate cyclase activation, we used Gpp(NH)p, since this GTP anaolg is not hydrolyzed at the regulatory site (13). As shown in Fig. 5, activation of adenylate cyclase by isoproterenol plus Gpp(NH)p showed the same initial rate both in toxin-treated and untreated preparations. The rate of activation, however, leveled off earlier in the toxin-treated preparation, and prolonged periods of incubation with Gpp(NH)p and iso-

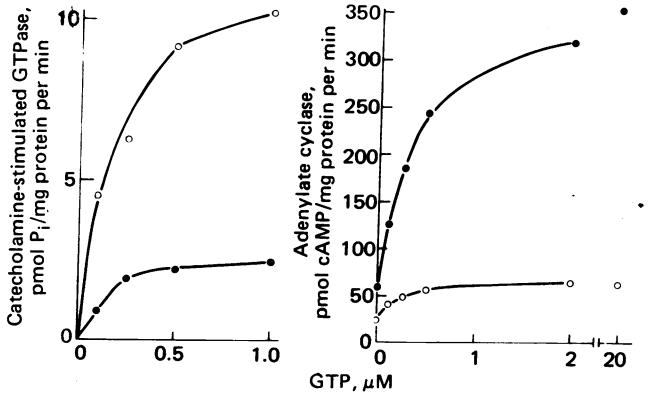

FIG. 3. Toxin effect on the catecholamine-stimulated GTPase (*Left*) and adenylate cyclase (*Right*) activities at various GTP concentrations. Membranes pretreated in the absence (○) or presence (●) of toxin (6 μg/ml) were assayed for GTPase and adenylate cyclase activities at the indicated GTP concentrations. Adenylate cyclase was assayed in reaction mixture II in the presence of 50 μM isoproterenol. GTPase activity was assayed in the presence of 1 mM App(NH)p.

Table 2. Effect of cholera toxin on activation of the adenylate cyclase by GTP and Gpp(NH)p

Additions	Adenylate cyclase, pmol cAMP/mg protein per min	
	No toxin	Toxin
None	2	11
Isoproterenol	27	57
GTP	2	44
GTP + isoproterenol	68	404
Gpp(NH)p	19	70
Gpp(NH)p + isoproterenol	415	314

Membranes were pretreated in the absence or presence of toxin (6 μg/ml) as described in *Materials and Methods*. Adenylate cyclase was assayed in reaction mixture II. The concentration of isoproterenol was 50 μM; that of the guanyl nucleotides, 100 μM.

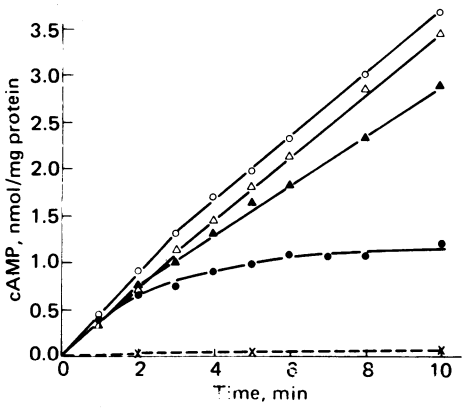

FIG. 4. Toxin effect on refractoriness to propranolol of adenylate cyclase preactivated by hormone plus guanyl nucleotide. Membranes were treated in the absence or presence of toxin (6 μg/ml). After this treatment the membranes (1 mg/ml) were incubated for 3 min at 37° in a medium containing 10 mM Hepes buffer (pH 7.4), 2 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 5 mM phospho*enol*pyruvate, 50 μg of pyruvate kinase per ml, and 10 μg of myokinase per ml. Isoproterenol was present in all systems; GTP or Gpp(NH)p, each at 100 μM, was added as indicated. At time zero the activated membranes were added to an equal volume of adenylate cyclase reaction mixture II at 37° in which propranolol (50 μM) was either present or absent, as indicated. At the times shown on the abscissa, the reaction was stopped and the amount of cAMP formed was determined. No toxin: ×, GTP then propranolol. Toxin present: ○, GTP; ●, GTP then propanolol; △, Gpp(NH)p; ▲, Gpp(NH)p then propranolol.

proterenol resulted in some decline in adenylate cyclase activity.

DISCUSSION

The present studies on cholera toxin lend support to the view that the guanyl nucleotide binding component of the adenylate cyclase system plays a dual role. On the one hand, in the presence of hormone plus GTP it activates the adenylate cyclase; on the other hand, it functions in the hydrolysis of bound GTP and thus terminates the activation (Fig. 6). (The latter reaction is henceforth referred to as the turn-off GTPase reaction.) It is therefore obvious that any decrease in the rate of hydrolysis of GTP at the regulatory site would maintain more of the enzyme in the active state. Since cholera toxin inhibits the turn-off GTPase, it is most likely that it increases adenylate cyclase activity by the above mechanism.

The finding that cholera toxin enhances stimulation of the adenylate cyclase by hormone (18–21) is in accord with the following model of hormone action. The adenylate cyclase in the presence of hormone plus GTP continuously oscillates between the inactive and the active states and therefore reveals only a fraction of its maximal catalytic activity. The role of the hormone is thus to repeatedly generate the active adenylate cyclase–GTP complex (Fig. 6). Several lines of evidence support this mechanism of hormone action. The hormone enhances the rate of adenylate cyclase activation by Gpp(NH)p. Once the enzyme had been activated by this hydrolysis-resistant analog it remained in the active state even when the hormone receptor was blocked by propranolol (11). It was therefore concluded that the hormone receptor functions in the activation of the enzyme by the nucleotide. Since GTP, in contrast to Gpp(NH)p, is hydrolyzed by the turn-off GTPase, the hormone must continuously regenerate the active adenylate cyclase–GTP complex in order to maintain enzyme activity. This mechanism implies that the hormone acts indirectly to enhance the GTPase activity, as was indeed found by direct measurements (12). When the GTPase was partially inhibited by cholera toxin, GTP behaved

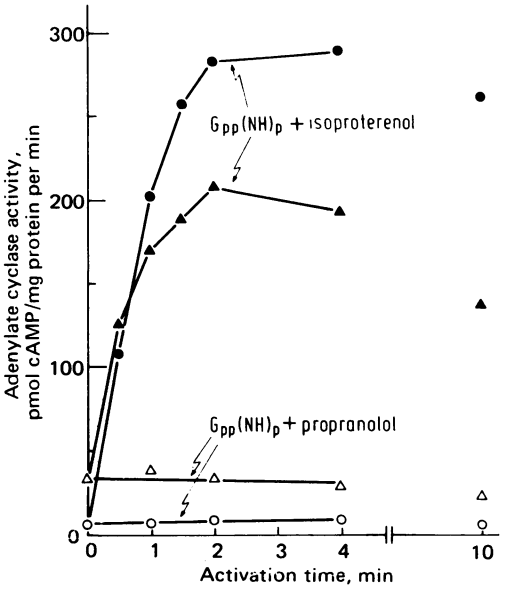

FIG. 5. Time course of adenylate cyclase activation by isoproterenol plus Gpp(NH)p in toxin-treated and untreated membranes. Membranes were treated in the absence (circles) or presence (triangles) of 6 μg of toxin per ml. The membrane preparations (1 mg/ml) were warmed to 37° in a medium containing 10 mM Hepes buffer (pH 7.4), 2 mM $MgCl_2$, and 2 mM 2-mercaptoethanol. At time zero activation of the adenylate cyclase was initiated by the addition of isoproterenol and Gpp(NH)p to give concentrations of 10 μM and 100 μM, respectively. Control systems received Gpp(NH)p and propranolol (100 μM and 1 μM, respectively). At the times shown on the abscissa, aliquots were removed and adenylate cyclase activity was determined in reaction mixture II. Assay systems containing 50 μM propranolol were used to prevent further activation by Gpp(NH)p during the adenylate cyclase assay (10 min at 37°).

like Gpp(NH)p. Activation by hormone plus GTP produced a higher adenylate cyclase activity after toxin treatment and the activated enzyme became partially refractory to subsequent addition of propranolol. This again indicates that the life span of GTP at the regulatory site determines the adenylate cyclase activity.

Activation of the adenylate cyclase by fluoride apparently does not require GTP (7). The effect of cholera toxin on fluoride activation of the enzyme is not understood. It should be noted, however, that somewhat higher concentrations of toxin were required to block the fluoride effect than those necessary to

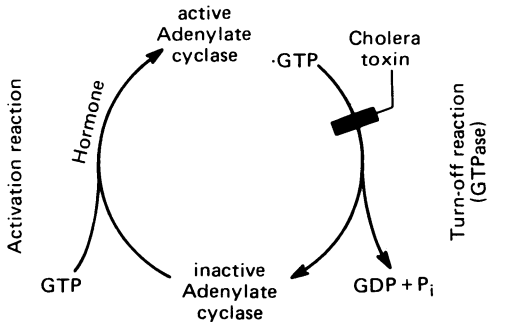

FIG. 6. Model of regulation of adenylate cyclase activity. Conversion of the adenylate cyclase from an inactive to an active state requires hormone-induced introduction of GTP to the regulatory site. The turn-off reaction occurs by hydrolysis of the bound GTP. Continuous hydrolysis of GTP depends on concurrent operation of both the activation and the turn-off reactions, and is therefore hormone dependent. Cholera toxin inhibits the turn-off reaction and has no effect on the activation reaction.

produce maximal activation of the adenylate cyclase by hormone and GTP.

It should be stressed that the mechanism of cholera toxin action, as proposed in the present study, is based on findings that were confined to adenylate cyclase from turkey erythrocytes. In this system we have developed conditions to measure a hormone-stimulated hydrolysis of GTP at the regulatory site. GTPase activity in other adenylate cyclase systems has not yet been measured due to the very high background of nonspecific nucleotide triphosphatases (12). Nevertheless, a general turn-off GTPase mechanism is strongly indicated since, in all of the hormone-responsive adenylate cyclase systems so far tested, cholera toxin enhanced both the basal and hormone-stimulated activities and the hydrolysis-resistant analog Gpp(NH)p caused maximal and persistent activation of the enzyme. It is anticipated that in toxin-treated adenylate cyclase systems other than that of the turkey erythrocyte, GTP will also prove to be as efficient an activator of the adenylate cyclase as Gpp(NH)p. Taken together, the present findings indicate that regulation of adenylate cyclase activity can be achieved not only by controlling the "on" rate through the hormone receptor, but also by controlling the "off" rate through the GTPase reaction. It is possible that, in addition to cholera toxin, other agents, both cellular and synthetic, will be found to act through the turn-off GTPase mechanism.

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