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Reconstitution of cholera toxin-activated adenylate cyclase

(S49 cyc⁻ variants/guanine nucleotide regulation/complementation analysis)

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ABSTRACT **Reconstitution of adenylate cyclase activity responsive to stimulation by guanylyl-5'-imidodiphosphate or NaF may be achieved by mixing dilute Lubrol 12A9-solubilized extracts of wild-type S49 membranes with membranes of an adenylate cyclase-deficient variant. Experiments using *N*-ethylmaleimide to inactivate components of the adenylate cyclase system indicate that distinct components from both wild-type detergent extracts and adenylate cyclase-deficient membranes are essential for reconstitution. These results and conclusions confirm those of E. M. Ross and A. G. Gilman [*J. Biol. Chem.* (1977) 252, 6966–6969]. Detergent extracts of cholera toxin-treated wild-type membranes yield a reconstituted adenylate cyclase as responsive to GTP as to guanylyl-5'-imidodiphosphate whereas, in the absence of cholera toxin treatment, GTP has little or no effect. Cholera toxin-treated adenylate cyclase-deficient membranes and Lubrol 12A9 extracts from them, however, fail to yield a reconstituted adenylate cyclase that responds to GTP with an increase in cyclase activity. Because treatment of the adenylate cyclase-deficient variants with cholera toxin is without effect on the reconstituted cyclase, we propose that the cholera toxin substrate is absent or altered in the adenylate cyclase-deficient phenotype.**

Cholera toxin activates adenylate cyclase in a wide variety of animal cells (1). Recent observations suggest that the toxin may act by modifying a guanyl nucleotide regulatory site associated with the enzyme. In mammalian (2) and avian (3) plasma membranes, cholera toxin markedly increases the sensitivity of adenylate cyclase to stimulation by GTP but not to stimulation by guanylyl-5'-imidodiphosphate [Gpp(NH)p] and other hydrolysis-resistant GTP analogs. Cassel and Selinger(3) recently reported that toxin treatment inhibits a hormone-stimulated GTPase in turkey erythrocyte membranes. With purified plasma membranes, NAD^+ must be present for cholera toxin's effect on adenylate cyclase (2–5). The toxin has also been shown to have NADase activity (6) and can catalyze the transfer of the ADP-ribose moiety of NAD^+ to arginine (7). Taken together, these observations suggest that cholera toxin's action on cells involves a similar enzymatic ADP-ribosylation of a protein substrate identical to or closely associated with the guanyl nucleotide regulatory component of adenylate cyclase.

As a first step in defining the site of action of cholera toxin, we have taken advantage of an *in vitro* complementation system (8) in which separate components of hormone-sensitive adenylate cyclase can be recombined to produce hormone- and guanyl nucleotide-regulated activity *in vitro*. This complementation system uses membrane fractions prepared from a phenotypic variant, termed cyc⁻, of the S49 mouse lymphoma cell (9). Unlike that of the wild-type S49 parent, adenylate cyclase in cyc⁻ membranes fails to respond to stimulation by hormones, guanyl nucleotides, and fluoride ion (8, 9). When detergent-solubilized extracts of wild-type membranes are added to cyc⁻ membranes, responsiveness to these agents is reconstituted (8). We have used this procedure to examine both the reconstitution of responsiveness to cholera toxin and the possible lesion responsible for the cyc⁻ phenotype.

METHODS

Cell Culture. Wild-type and cyc⁻ S49 lines were grown as described (10) in Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum.

Isolation and Solubilization of Plasma Membranes. S49 cell plasma membranes were prepared by a modification (2) of the method described by Ross *et al.* (11). Membranes were stored at −70° with no loss of either hormone or cholera toxin responsiveness for at least 3 months. Solubilization of plasma membranes was accomplished by the addition of Lubrol 12A9 to a final concentration of 0.7% to a membrane solution (2–3.5 mg of protein per ml) in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)/100 μM GTP/2 mM $MgCl_2$/1 mM EDTA/1 mM 2-mercaptoethanol, pH 8.0. The solution was allowed to stand on ice for 30 min with periodic agitation and then was centrifuged at 100,000 × *g* for 30 min.

Reconstitution of Donor Extracts and Recipient Membranes. Reconstitution of the 100,000 × *g* supernatant fraction of detergent extracts with recipient membranes was accomplished essentially as described by Ross and Gilman (9). Extracts were added gradually over a 5-min period to recipient membranes in 20 mM Hepes/2 mM $MgCl_2$/1 mM EDTA/1 mM 2-mercaptoethanol, pH 8.0, and allowed to stand on ice for 30 min. Generally, detergent extract was diluted a minimum of 1:6 into the reconstitution system containing recipient membranes. The reconstituted system was then diluted an additional 1:3 to 1:5 when added to the adenylate cyclase assay mixture.

Assay of Adenylate Cyclase Activity. Adenylate cyclase activity was determined as described (2). The reaction was stopped by the addition of acetic acid to a final concentration of 50 mM, and cyclic AMP was determined by the competitive protein-binding method (12).

Cholera Toxin Treatment. S49 cells were incubated in normal growth medium containing cholera toxin (100 ng/ml) for 17 hr prior to the preparation of plasma membranes. Alternatively, the plasma membranes were treated with cholera toxin (100 μg/ml) (preactivated with 20 mM dithiothreitol) and NAD^+ (2.5 mM) for 10 min at 30° (2). Similar results were obtained with both preparations.

Materials. Lubrol 12A9 (previously referred to as Lubrol PX)

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Abbreviations: cyc⁻, adenylate cyclase-deficient S49 cell variants; Gpp(NH)p, guanylyl-5'-imidodiphosphate; NEM, *N*-ethylmaleimide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Table 1. Sensitivity of wild-type detergent extracts and cyc⁻ membranes to *N*-ethylmaleimide

Reconstituted system		Adenylate cyclase, pmol/10 min/mg donor extract			% of control activity		
Donor extract	Recipient membranes	GTP	Gpp(NH)p	NaF	GTP	Gpp(NH)p	NaF
		Experiment 1					
Wild-type	cyc⁻	—	440	420	—	100	100
Wild-type + 3 mM NEM	cyc⁻	—	360	340	—	82	81
Wild-type	cyc⁻ + 10 mM NEM	—	100	140	—	23	33
Wild-type + 3 mM NEM	cyc⁻ + 10 mM NEM	—	0	0	—	0	0
		Experiment 2					
Wild-type	cyc⁻	—	710	755	—	100	100
Wild-type	cyc⁻ + 10 mM NEM	—	50	100	—	7	13
Wild-type$_{CT}$	cyc⁻	1920	1840	1530	100	100	100
Wild-type$_{CT}$	cyc⁻ + 10 mM NEM	190	100	275	10	5	18

cyc⁻ membranes or detergent extracts from wild-type membranes were incubated with the indicated concentration of *N*-ethylmaleimide (NEM) for 20 min on ice. The reaction was stopped by addition of an equimolar amount of dithiothreitol, and the appropriate wild-type extract and cyc⁻ membranes were mixed. The reconstituted system was assayed in the presence of 100 μM GTP, 100 μM Gpp(NH)p, or 10 mM NaF. The protein concentration in each 0.1-ml assay was 10 μg and 16 μg for donor extract and 30 μg and 10 μg for cyc⁻ membranes in exp. 1 and 2, respectively. Cholera toxin-treated preparations (designated CT) were prepared from toxin-treated cells. Values represent the mean of triplicate determinations which were within 10% of the mean. Each experiment is representative of two independent experiments.

was a gift from I.C.I., Ltd., Gpp(NH)p was purchased from Boehringer Mannheim, GTP was from Sigma, and cholera toxin was from Schwarz/Mann.

RESULTS AND DISCUSSION

Measurement of Cholera Toxin Effect. In partially purified S49 membranes, β-adrenergic amines and prostaglandins stimulate adenylate cyclase only in the presence of a guanyl trinucleotide, such as GTP or its less hydrolyzable analog, Gpp(NH)p. Gpp(NH)p alone can stimulate the S49 cyclase, whereas GTP by itself produces little or no effect. After the membranes are exposed to cholera toxin plus NAD^+, however, adenylate cyclase is rendered as responsive to GTP as to Gpp(NH)p (2). Thus, the enhanced ability of GTP alone to activate adenylate cyclase is a measure of cholera toxin's effect on the cyclase system. Accordingly, we have expressed the results of some reconstitution experiments in terms of the ratio of adenylate cyclase activity in the presence of GTP to that in the presence of Gpp(NH)p. This ratio is a measure of cholera toxin's effect, normalized for the stimulation produced by Gpp(NH)p.

Characterization of Reconstitution. Detergent-solubilized extracts of wild-type membranes at relatively high protein concentrations (>0.2–0.3 mg/ml) contained adenylate cyclase responsive to stimulation by Gpp(NH)p and NaF. When aliquots of these detergent extracts were diluted to a protein concentration of 0.02–0.10 mg/ml, essentially no cyclase activity was measurable in response to these effectors. This result is consistent with the notion that two or more dissociable components are required for effector-stimulated enzyme activity (13). When these diluted extracts were mixed with cyc⁻ membranes, however, adenylate cyclase activity responsive to stimulation by Gpp(NH)p and NaF was reconstituted (Table 1).

That reconstitution requires a minimum of two distinct components, one contributed by wild-type donor extract and one by cyc⁻ recipient membranes, is indicated by experiments (Table 1) in which donor or recipient had previously been exposed to *N*-ethylmaleimide (NEM). Treatment of detergent extracts from wild-type membranes with NEM decreased the reconstituted activity by less than 20%, whereas approximately 75% of the reconstituted activity was lost if the cyc⁻ recipient membranes had been exposed to NEM. The 25% of total ac-

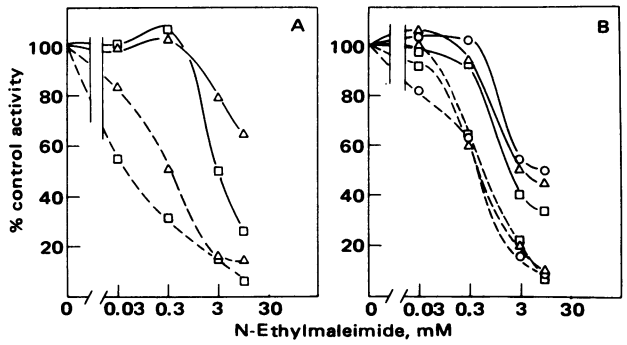

FIG. 1. Inactivation of adenylate cyclase components by NEM. Lubrol 12A9 extracts of control (*A*) or cholera toxin-treated (*B*) membranes were incubated with the indicated concentrations of NEM for 20 min on ice. The reaction was terminated by addition of equimolar amounts of dithiothreitol, and aliquots were used for either measurement of adenylate cyclase activity in the extract (broken lines) or mixed with cyc⁻ membranes (solid lines). The extract or reconstituted system was then assayed in the presence of 100 μM GTP (○), 100 μM Gpp(NH)p (△), or 10 mM NaF (□). Adenylate cyclase activity of the extract, in the absence of NEM treatment, for control membranes with Gpp(NH)p or NaF was 200 and 220 pmol/10 min per mg of extract protein. For extracts of toxin-treated membranes, cyclase activity in the presence of GTP, Gpp(NH)p, or NaF was 320, 340, or 250 pmol/10 min/mg extract protein, respectively. The extract protein concentration in each 0.1-ml assay was 30 μg and 35 μg for control and toxin-treated preparations, respectively. Cyclase activity in the absence of NEM treatment, in the reconstituted system of the control (*A*) was 1100 or 1800 pmol/10 min per mg of extract protein in the presence of GTP or Gpp(NH)p; for the toxin-treated preparation (*B*), activities were 600, 600, or 350 pmol/10 min per mg of extract protein for GTP, Gpp(NH)p, or NaF, respectively. The protein concentration of the reconstituted system was 30 μg for cyc⁻ membranes and 11 μg for control or 15 μg for toxin-treated extract per 0.1-ml assay volume. Values represent the mean of triplicate determinations which were within 10% of the mean. Each experiment is representative of three independent experiments.

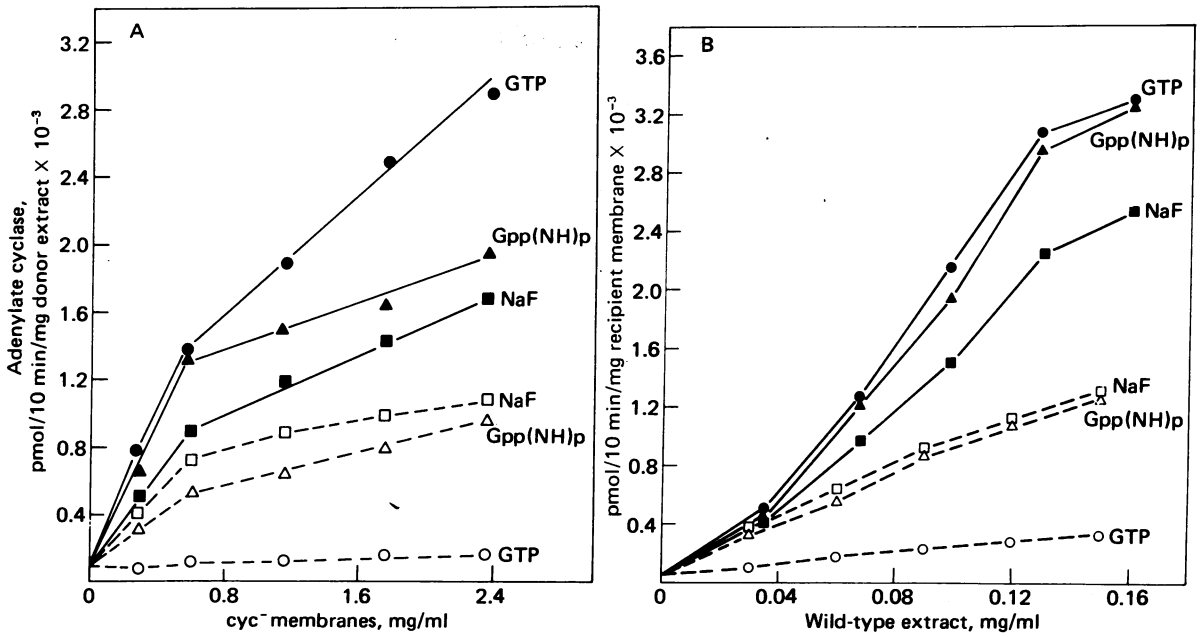

FIG. 2. Concentration dependence of detergent extracts from wild-type membranes and cyc⁻ recipient membranes for reconstitution of adenylate cyclase activity. (*A*) Increasing concentrations of cyc⁻ membranes in 250 μl were mixed with 50 μl of wild-type Lubrol 12A9 extract. Detergent extracts were prepared from cholera toxin-treated (solid lines) or control (broken lines) membranes. Aliquots (20 μl) were removed for assay of adenylate cyclase activity in the presence of 100 μM GTP (●, ○), 100 μM Gpp(NH)p (▲, △), or 10 mM NaF (■, □). Each 0.1-ml assay volume contained 10 μg of wild-type extract. (*B*) Increasing concentrations of detergent extracts from wild-type membranes in 50 μl were mixed with 250 μl of cyc⁻ membranes. The Lubrol 12A9 concentration was constant for all extract protein concentrations. Wild-type extract was prepared from toxin-treated (solid lines) or control (broken lines) membranes. Aliquots (20 μl) were removed for assay of cyclase activity in the presence of 100 μM GTP (●, ○), 100 μM Gpp(NH)p (▲, △), or 10 mM NaF (■, □). Each 0.1-ml assay volume contained 10 μg of cyc⁻ membranes.

tivity observed in reconstitution with NEM-treated cyc⁻ membranes represents an upper limit for the catalytic cyclase activity contributed by detergent extracts from wild-type membranes under these conditions of reconstitution. However, when both cyc⁻ membranes and detergent extracts from wild-type membranes were treated with NEM, all reconstituted activity was lost, indicating that a distinct component from each is essential for reconstitution. A similar requirement for functional cyc⁻ membranes was found for reconstitution of cholera toxin-activated adenylate cyclase (Table 1, Exp. 2).

The fact that at least two components are required for expression of adenylate cyclase activity is additionally supported by the differential sensitivities to NEM of cyclase activity measured directly in detergent extracts of wild-type membranes as compared with their ability to reconstitute activity when mixed with cyc⁻ membranes (Fig. 1). Adenylate cyclase activity in the extract was at least 50-fold more sensitive to NEM inactivation than was the ability to reconstitute Gpp(NH)p and NaF responsiveness when the extracts were mixed with cyc⁻ membranes (Fig. 1*A*). With extracts prepared from toxin-treated cells, this difference in sensitivity to NEM was slightly decreased (Fig. 1*B*). Both the detergent extract from toxin-treated cells and the reconstituted system contained GTP-active cyclase whose NEM-sensitivity parallelled that observed with Gpp(NH)p and NaF.

In some experiments, cyclase responsiveness to NaF showed a tendency to inhibition by lower concentrations of NEM than are required to inhibit Gpp(NH)p responsiveness (e.g., Fig. 1*A*). This observation remains unexplained, although it raises the possibility that NEM may be inactivating more than one component involved in reconstituting cyclase responsiveness.

Effector-stimulated activity in the reconstituted mixture depended upon the concentrations of cyc⁻ recipient membranes and donor extract (Fig. 2). When a fixed concentration of wild-type donor extract was used and the concentration of cyc⁻ membranes was varied, reconstituted cyclase activity appeared to approach a maximum, indicating that the concentration of wild-type extract is limiting (Fig. 2*A*). Cyclase activity in the presence of GTP appeared to be less saturable than Gpp(NH)p- or NaF-stimulated activities. The reason for this difference is unknown. In the reciprocal experiment (Fig. 2*B*), reconstituted activity failed to reach a maximum, indicating that even the relatively low concentration of cyc⁻ membranes used (0.1 mg/ml) was not limiting. In both experiments, wild-type extract from cholera toxin-activated donor membranes produced markedly increased responsiveness of the reconstituted mixture to GTP, relative to the changes in responsiveness to Gpp(NH)p and NaF.

These findings are in agreement with the similar reconstitution experiments of Ross and Gilman (8, 13) and support their hypothesis that a minimum of two distinct components—one of which is contributed by cyc⁻—is required for activation of adenylate cyclase by guanyl nucleotides and fluoride ion. Furthermore, our results demonstrate that the increased GTP responsiveness characteristic of wild-type membranes activated by cholera toxin is expressed in the reconstituted system.

Complementation Analysis of Cholera Toxin Action. Extracts of wild-type membranes from cells treated with cholera toxin conferred on the reconstituted adenylate cyclase system the increased sensitivity to GTP characteristic of toxin-activated cyclase (Table 1; Figs. 1 and 2). The fact that cholera toxin's effect persisted throughout the extraction and reconstitution procedures allowed us to ask whether cyc⁻ membranes treated with toxin can similarly reconstitute the toxin's effect on adenylate cyclase activation by GTP.

If cholera toxin's substrate or site of action is normal in cyc⁻ membranes, then the mixing of detergent extracts or membranes of toxin-treated cyc⁻ preparations with the appropriate

Table 2. Complementation analysis of reconstituted cholera toxin-activated adenylate cyclase

Donor extract	Recipient membrane	Adenylate cyclase, pmol/10 min/mg recipient membrane			
		GTP	Gpp(NH)p	NaF	GTP/Gpp(NH)p
		Experiment 1			
Wild-type$_{CT}$	cyc$^-$	480	375	265	1.25
Wild-type	cyc$^-$	60	421	570	0.14
cyc$^-_{CT}$	cyc$^-$	40	50	30	—
cyc$^-$	cyc$^-$	40	30	25	—
cyc$^-_{CT}$	Wild-type	240	2070	2000	0.12
cyc$^-$	Wild-type	215	1440	1700	0.15
		Experiment 2			
Wild-type	cyc$^-_{CT}$	60	520	525	0.12
Wild-type	cyc$^-$	70	480	580	0.15
cyc$^-$	cyc$^-_{CT}$	30	40	20	—
cyc$^-$	cyc$^-$	35	30	20	—
cyc$^-$	Wild-type$_{CT}$	880	810	510	1.10
cyc$^-$	Wild-type	120	1220	1000	0.10

Membrane preparations of wild-type or cyc$^-$ cells incubated with cholera toxin (CT), or without it, were used in the reconstituted system as either donor extract (Exp. 1) or recipient membranes (Exp. 2). Aliquots from the reconstituted system were assayed in the presence of 100 μM GTP, 100 μM Gpp(NH)p, or 10 mM NaF. The protein concentrations in each 0.1-ml assay volume were: for Exp. 1, 14 μg wild-type and 16 μg cyc$^-$ recipient membranes, and 4 μg wild-type$_{CT}$, 3 μg wild-type, 5 μg cyc$^-_{CT}$, and 4 μg cyc$^-$ donor extract; for Exp. 2, 5 μg wild-type and 5 μg cyc$^-$ donor extract, and 15 μg wild-type$_{CT}$, and 20 μg wild-type, 19 μg cyc$^-_{CT}$, and 22 μg cyc$^-$ recipient membranes. Values represent the mean of triplicate determinations which were within 10% of the mean. Each experiment is representative of at least two independent experiments.

wild-type preparation should reconstitute the increased GTP/Gpp(NH)p stimulation ratio characteristic of the toxin's activation of adenylate cyclase. Conversely, if the cholera toxin substrate is altered or absent in cyc$^-$, toxin treatment of cyc$^-$ should not affect this ratio, measured in the reconstituted system. To prevent any effect of cholera toxin on the wild-type toxin substrate when toxin-treated cyc$^-$ preparations were used, these experiments were always conducted using membranes prepared from toxin-treated cells rather than membranes treated with toxin and NAD$^+$. The results in Table 2 indicate that adenylate cyclase in the reconstituted mixture shows increased sensitivity to GTP [as compared with Gpp(NH)p] only if the wild-type component of the mixture has been previously treated with toxin. Preparations from toxin-treated cyc$^-$ cells failed to increase GTP sensitivity in the reconstitution mixture, whether the cyc$^-$ acted as the detergent-solubilized donor (Exp. 1) or membrane recipient (Exp. 2).

Toxin Action and the Guanyl Nucleotide Regulatory Site. Our experiments indicate that the site of action or substrate of cholera toxin is functionally defective in membranes of the cyc$^-$ variant cell and that this component can be supplied by detergent-solubilized extracts of wild-type membranes. The extracts from wild-type membranes supply a factor or factors, altered or missing in cyc$^-$, that mediate stimulation of adenylate cyclase by guanyl nucleotides, fluoride ion, and β-adrenergic amines (8).

The number and molecular nature of wild-type factors responsible for reconstitution remain undefined. However, our findings are consistent with the notion that these factors include the putative substrate of cholera toxin, which may be identical to the guanyl nucleotide regulatory site of adenylate cyclase. This notion is supported by kinetic and biochemical evidence obtained in S49 and other systems: GTP and Gpp(NH)p appear to occupy the same regulatory site (2, 3, 14) and cholera toxin treatment makes adenylate cyclase respond to GTP as if it were the less hydrolyzable analog (2, 3). Additionally, cholera toxin treatment decreases the activity of a specific hormone-sensitive GTPase in turkey erythrocyte membranes (3), and this GTPase is inhibited by Gpp(NH)p (14). These observations suggest that the GTPase acts as a "turn-off" mechanism for adenylate cyclase.

If extracts from wild-type membranes contribute a guanyl nucleotide regulatory site to the reconstituted system, what is the component contributed by cyc$^-$? Ross and Gilman (13) have suggested that the cyc$^-$ contribution may be catalytic adenylate cyclase. In recent experiments they have also found that cyc$^-$ membranes express adenylate cyclase activity in the presence of Mn^{2+} and that NEM inactivation of this Mn^{2+}-sensitive activity prevents reconstitution of Gpp(NH)p- and NaF-stimulated cyclase in the reconstituted mixture (15). We have confirmed these results in our laboratory (data not shown).

Based on this evidence, we propose a working hypothesis (Fig. 3) for the regulation of adenylate cyclase by guanyl nucleotides and cholera toxin. At least two separable components are required: the guanyl nucleotide regulatory site (N) and catalytic adenylate cyclase (C). The cyclase activity of C alone is low; increased conversion of ATP to cyclic AMP occurs when C combines in a ternary complex with N and a guanyl trinu-

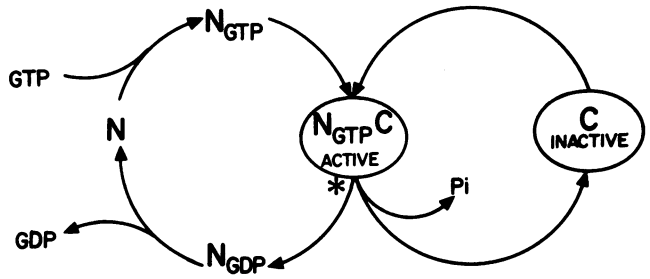

FIG. 3. Two-component model for guanine nucleotide regulation of adenylate cyclase activity. The guanine nucleotide regulatory component (N) is contributed by wild-type extracts, and catalytic adenylate cyclase (C) is contributed by cyc$^-$ membranes in the reconstituted system. *, Site of toxin action. See text for discussion.

cleotide (N_{GTP}·C). This ternary complex possesses intrinsic GTPase activity; hydrolysis of the bound GTP serves to terminate cyclic AMP synthesis by the complex and results in dissociation of N_{GDP} and C. Our hypothesis is kinetically identical to the model proposed by Cassel and Selinger (3). However, we can now add to the model a minimum number of components required for cyclase activation.

Cassel and Selinger (3) attributed the activation of adenylate cyclase by cholera toxin to inhibition of the GTPase "turn-off" mechanism. It is attractive to predict that cholera toxin will be found to modify N enzymatically, possibly by an ADP-ribosylation reaction. The ADP-ribosylated N will bind GTP and associate with and activate C just as unmodified N does, but its GTPase activity in the ternary complex will be decreased. In this scheme, the role of the cholera toxin substrate is closely analogous to that of elongation factor 2 in protein synthesis. Like the hypothetical N, elongation factor 2 is a GTP-binding protein that acquires GTPase activity when associated with another molecular complex (mRNA, ribosome, and aminoacyl-tRNA) (16, 17). ADP-ribosylation of elongation factor 2, catalyzed by the A fragment of diphtheria toxin, blocks protein synthesis by specifically inhibiting the factor's ribosome-dependent GTPase activity (17, 18).

At present the predictive force of this analogy rests largely on the structural, functional, and enzymatic similarities between diphtheria and cholera toxins, including their mutual requirements for NAD^+ for action in cell-free systems and ADP-ribosylation reactions catalyzed by both toxins *in vitro*. To test the prediction in detail will require resolution and characterization of the cholera toxin substrate. The reconstitution system we describe provides an assay for the cholera toxin substrate that may prove useful in its purification.

Finally, none of our results excludes the participation of additional components necessary for expression of adenylate cyclase activity. For example, NaF does not require GTP to activate cyclase (11) and may work by affecting N or a separate component (19, 20). As previously observed (3), cholera toxin decreases NaF activation while it increases GTP activation of adenylate cyclase (Table 2; Fig. 2). If the cyc^- lesion affects the guanine nucleotide regulatory component, it also affects some component involved in activation of adenylate cyclase by NaF. Further investigation will be necessary to define the number of such components and their precise relation to the site of action of cholera toxin.

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