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REGULATORY STATES OF ADENYLATE CYCLASE IN RL-PR-C CLONED RAT HEPATOCYTES *

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Summary

The adenylate cyclase of cloned differentiated rat hepatocytes (RL-PR-C) is regulated by cholera toxin, guanine nucleotides and fluoride. The activation of hepatic adenylate cyclase by cholera toxin was additive with that by GTP and synergistic with that by epinephrine.

In contrast, when membranes were exposed to cholera toxin in the presence of Gpp(NH)p or fluoride, the response was the same as to these agents in the absence of cholera toxin.

Cholera toxin-activated membranes were responsive only to epinephrine and GTP, while fluoride-activated membranes responded somewhat to all other agents, and Gpp(NH)p-activated membranes responded to no other agents.

These data suggest that responsiveness of hepatic adenylate cyclase to cholera toxin, fluoride and Gpp(NH)p cannot be expressed simultaneously. A model is presented to explain these observations which invokes multiple states of adenylate cyclase, each being sensitive to, or brought about by, a different regulatory agent.

Introduction

Genetic [1—3] and biochemical [4—7] evidence indicates that hormone-sensitive adenylate cyclase consists of at least three separate components; a hor-

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mone receptor, a catalytic unit which converts ATP to cyclic AMP, and one or more regulatory factors. At least one of these regulatory factors seems to mediate effects by guanine nucleotides [8—10]. The findings that β-agonists increase membrane GTPase activity [11] and enhance displacement of bound GDP by GTP in avian erythrocytes [12] have led Cassel and Selinger [11] to propose that the hydrolysis of GTP serves as the ultimate signal to terminate adenylate cyclase activity, which is active when GTP is bound to the regulatory site.

Cholera toxin, which activates adenylate cyclase in a wide variety of cell types [13], modifies regulation by guanine nucleotides [14,15] and inhibits catecholamine-stimulated GTPase activity [14—16]. Furthermore, cholera toxin has been found to ADP-ribosylate a specific membrane protein in a number of cell types [17—21]. This ADP-ribosyl acceptor of pigeon erythrocytes is also capable of binding GTP, and is thought to be responsible for guanine nucleotide and fluoride sensitivity in this system [22]. This same protein was reported to be absent in a phenotypic variant of S49 cells which is unresponsive to these regulators [18]. These observations suggest that cholera toxin covalently modifies one or more proteins involved in the regulation of adenylate cyclase by guanine nucleotides, hormones and fluoride.

We have been studying a chromosomally and culturally stable cloned line (RL-PR-C) of rat hepatocytes [23], of which the adenylate cyclase is sensitive to epinephrine, guanine nucleotides, fluoride and cholera toxin [24]. Further, cholera toxin catalyzes the ADP-ribosylation of a single protein (55 000 daltons) of RL-PR-C cell membranes [21]. The present study examines the effect of cholera toxin modification on the regulation of adenylate cyclase activity of RL-PR-C hepatocytes and extends other studies of such regulation by examining interrelationships among other known activators of the enzyme. These studies demonstrate that adenylate cyclase activity of hepatocytes is not regulated solely by guanine nucleotides, as proposed by Cassel and Selinger [16], but more likely involves at least four separate, mutually exclusive states, the equilibrium of which depends on the activator ligands present.

Materials and Methods

Materials. Cholera toxin was obtained from Schwarz-Mann. GTP, Gpp(NH)p and D-epinephrine were obtained from Sigma. Stock solutions of 50 mM epinephrine were prepared in 0.1 N HCl each day and diluted in buffer just prior to assay. GTP solutions were also made fresh daily. Adenosine 5′-[α-^{32}P]triphosphate (400—500 Ci/mmol) was obtained from Amersham.

Cell-culture. RL-PR-C hepatocytes were grown in monolayer in Ham's F12 medium with 10% fetal bovine serum. The characteristics of this cell line have been described [23]. Cells utilized for these experiments were passage 21—25 (84—97 population doublings) and thus had not yet undergone spontaneous transformation (23,24]. Confluent monolayers of RL-PR-C cells were dislodged with trypsin [25] and seeded into T150 flasks (Costar). The medium was changed every 48 h, and 16 h prior to each experiment. During these 5 days in culture, the cells doubled approximately twice. The cells were just confluent at the time of assay.

Membrane preparation. Monolayers of hepatocytes were washed quickly at

4°C with phosphate-buffered saline (pH 7.4) and the cells scraped from the flask with a rubber policeman. The suspension in 1 mM $NaHCO_3$ was homogenized in a Potter-Elvehjem Teflon glass homogenizer at 4°C (10 strokes at 1000 rev./min). The homogenate was centrifuged at 4000 × *g*, the pellet washed once and suspended in 1 mM $NaHCO_3$ at a final concentration of 5—10 mg/ml protein.

Adenylate cyclase assay. Assay conditions were essentially those of Salomon et al. [26]. Final concentrations were as follows: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM cyclic AMP, 0.1% bovine serum albumin, 10 units creatine phosphokinase, 20 mM phosphocreatine, 0.5 mM [^{32}P]ATP (50 000 cpm/pmol), and 100—150 μg membrane protein, in a final volume of 100 μl. Where indicated, 1 mM NAD^+ and 2 mM dithiothreitol were also present. There was no phosphodiesterase inhibitor present, as we found this to be unnecessary with the membrane preparation employed.

Determination of cyclic AMP. Confluent monolayers of RL-PR-C cells were incubated for 10 min at 37°C with appropriate agents in 1.5% bovine serum albumin in phosphate-buffered saline (pH 7.5), as indicated in the figure legends. Monolayers were then washed quickly with cold phosphate-buffered saline, and the cells scraped from the dish into 1 ml 0.25% (v/v) acetic acid. Samples were boiled for 3 min, cooled on ice, and centrifuged twice at 2000 × *g*. The supernatant was assayed for cyclic AMP according to the method of Gilman [27].

Protein. This was determined by the method of Lowry et al. [28].

Results

Activation of adenylate cyclase. The adenylate cyclase of RL-PR-C hepatocyte membranes responded to known activators of the enzyme (Table I). Guanine nucleotides were maximally effective at 100 μM. At this concentration the enzyme was much more sensitive to Gpp(NH)p (4.3-fold activation) than to GTP (1.5-fold), suggesting that the hydrolysis of GTP plays some role in the regulation of adenylate cyclase activity by this agent.

TABLE I

ADENYLATE CYCLASE RESPONSIVENESS OF RL-PR-C HEPATOCYTE MEMBRANES

Adenylate cyclase activity was measured in the presence of 10 μg/ml cholera toxin. Values represent data from multiple experiments ± S.D. NAD^+ (1 mM) and dithiothreitol (2 mM) were present in all samples.

Additions	Adenylate cyclase activity (pmol cyclic AMP/10 min per mg protein)	
	Basal	+ Cholera toxin
None	600 ± 110	890 ± 50
GTP (100 μM)	864 ± 82	1120 ± 170
Gpp(NH)p (10 μM)	1560 ± 142	1708 ± 112
Gpp(NH)p (100 μM)	2586 ± 126	2632 ± 150
Epinephrine (5 μM)	967 ± 148	1269 ± 45
NaF (10 mM)	2599 ± 206	2628 ± 84

GTP (100 μM)

TABLE II

EFFECT OF GUANINE NUCLEOTIDES ON ADENYLATE CYCLASE ACTIVITY

Data are expressed as mean adenylate cyclase activity ± S.D. for triplicate determinations.

Additions	Adenylate cyclase activity (pmol cyclic AMP/10 min per mg protein)		
	Basal	GTP (100 μM)	Gpp(NH)p (10 μM)
None	395 ± 98	661 ± 87	1077 ± 107
GTP (100 μM)	641 ± 51	—	574 ± 8
Epinephrine (5 μM)	1172 ± 119	1445 ± 79	1936 ± 51
Cholera toxin (10 μg/ml)	649 ± 31	899 ± 115	1124 ± 83
NaF (10 mM)	1497 ± 135	1576 ± 83	1042 ± 115
Epinephrine + NaF	1580 ± 122	1572 ± 83	1141 ± 178

While adenylate cyclase responsiveness to epinephrine and NaF was maximal at 1 μM and 5 mM, respectively, the response to cholera toxin was linear between 0.06 and 1.2 μM (5 and 100 μg/ml). The response to cholera toxin was maximized by 1 mM NAD^+ and 2 mM dithiothreitol, so these reagents were routinely used when assaying cholera toxin responsiveness. Under these conditions, pretreatment of the toxin was not necessary. Basal adenylate cyclase activity varied between experiments, due perhaps to the quality of the [α-^{32}P]-ATP used for assay or to varying amounts of endogenous GTP being contributed by the membrane preparations. However, the magnitude of responses shown in Table I did not vary significantly between experiments. Adenylate cyclase activity was assayed for 10 min since accumulation of cyclic AMP was linear under all conditions during this time.

To examine the role of guanine nucleotides in the activation of adenylate cyclase of RL-PR-C hepatocytes, membranes were exposed to known activators of the enzyme in the presence and absence of GTP and Gpp(NH)p (Table II). When membranes were exposed to GTP and Gpp(NH)p simultaneously, the response was similar to that observed in the presence of GTP alone, suggesting that the affinity of this regulatory site is greater for GTP than for Gpp(NH)p. The stimulation of adenylate cyclase by epinephrine was additive with both guanine nucleotides, but stimulation by cholera toxin was additive with GTP only.

In contrast, the fluoride responsiveness of adenylate cyclase was not at all influenced by GTP. Further, the responses of adenylate cyclase to epinephrine and fluoride were not additive, and there was no enhancement of this epinephrine plus NaF response by guanine nucleotides. In fact, the response to NaF was the same in the presence or absence of epinephrine, GTP, or combinations of these agents. In contrast, when membranes were exposed to fluoride, fluoride plus epinephrine, or cholera toxin, in the presence of Gpp(NH)p, the response closely resembled that seen with Gpp(NH)p alone.

Actions of cholera toxin. We previously reported that cholera toxin catalyzes the ADP-ribosylation of a single 55 000 dalton membrane protein of RL-PR-C hepatocytes [21]. Although the role of this protein is not yet known, it is possible that it may be involved in one or more regulatory functions for adenylate

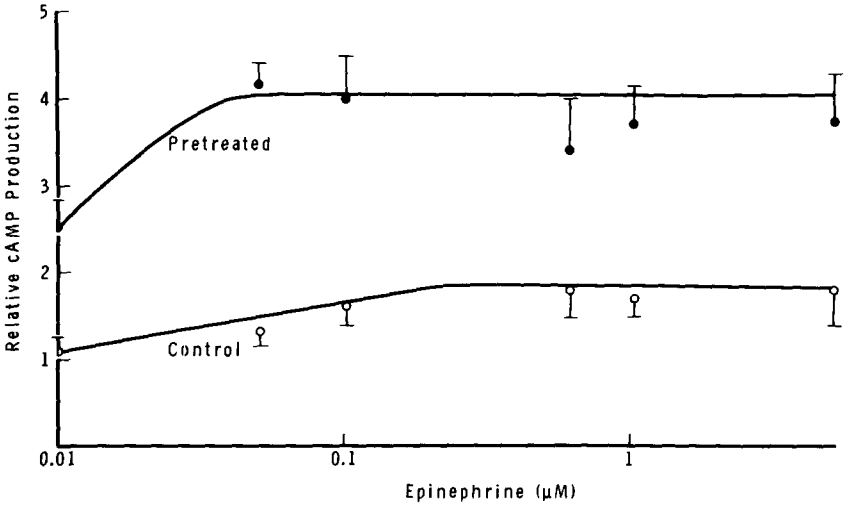

Fig. 1. Epinephrine responsiveness following cholera toxin pretreatment. Confluent monolayers of RL-PR-C hepatocytes were incubated with (●) or without (○) 5 μg/ml cholera toxin in phosphate-buffered saline, pH 7.4, for 3 h at 37°C. Monolayers were washed well at 22°C to remove excess cholera toxin, exposed to the indicated concentration of epinephrine in phosphate-buffered saline (pH 7.4)/1.5% bovine serum albumin for 10 min at 37°C, and cyclic AMP (cAMP) determined. Data is expressed as pmol cyclic AMP produced relative to control, in the absence of epinephrine, ±S.D. of triplicate determinations from two separate experiments. Control values were 2.58 and 29.7 pmol cyclic AMP/60 mm Petri dish for basal and cholera toxin-treated, respectively.

cyclase. To determine the role of cholera toxin's action on the regulation of adenylate cyclase, plasma membranes were exposed simultaneously to cholera toxin and other agents, and adenylate cyclase activity determined. The effect of cholera toxin was additive with those of GTP and epinephrine (Table I). In contrast, when adenylate cyclase activity was assayed with cholera toxin in the presence of fluoride or Gpp(NH)p, the response observed was that of these agents seen in the absence of cholera toxin. Thus, while the effects of cholera toxin and GTP were additive, those of Gpp(NH)p with cholera toxin and fluoride with cholera toxin were not.

When the effect of cholera toxin on epinephrine responsiveness was studied in intact cells, by measurement of cyclic AMP accumulation, a synergistic rather than additive effect of cholera toxin on epinephrine responsiveness was observed (Fig. 1). When monolayers of RL-PR-C hepatocytes were exposed to epinephrine after treatment with cholera toxin, the cyclic AMP response to low concentrations of epinephrine (0.01—1.0 μM) was greatly enhanced (4-fold compared to 2-fold with 0.1 μM epinephrine).

The effect of cholera toxin on adenylate cyclase was more pronounced in intact cells than in membrane preparations. For example, when intact cells were exposed to 5 μg/ml cholera toxin, cyclic AMP levels began to rise after 45 min and continued to increase for up to 4 h [29]; by 3 h, intracellular cyclic AMP levels were ten times higher than in control cells. In contrast, adenylate cyclase activity in a membrane preparation was activated by 100 μg/ml cholera toxin only 4.5-fold. Most likely, there is a requirement for intact membrane structure for the optimal action of cholera toxin on adenylate cyclase; alter-

TABLE III

EFFECTS OF CHOLERA TOXIN PRETREATMENT ON SUBSEQUENT ADENYLATE CYCLASE ACTIVITY

Confluent monolayers of hepatocytes were incubated with or without 5 μg/ml cholera toxin in phosphate-buffered saline (pH 7.4) for 3 h at 37°C, and membranes prepared. Adenylate cyclase was assayed in the presence of the additions shown. Values shown represent the means ± S.D. of triplicate determinations.

Additions	Adenylate cyclase activity (pmol cyclic AMP/10 min per mg protein)	
	Control	Cholera toxin-treated
Basal	550 ± 44	3509 ± 235
GTP (100 μM)	850 ± 50	3930 ± 200
Epinephrine (5 μM)	1503 ± 25	3629 ± 181
Epinephrine + GTP	2007 ± 121	4713 ± 190
Gpp(NH)p (100 μM)	2375 ± 142	2437 ± 112
NaF (10 mM)	2101 ± 100	2034 ± 189
GTP + NaF	2111 ± 194	2542 ± 89
Epinephrine + NaF	2199 ± 174	2027 ± 111
Epinephrine + NaF + GTP	2189 ± 221	2444 ± 235

natively, reagent concentrations with the membrane preparation are other than physiological (e.g. dissociation of the A fragment from the B subunits may not be complete).

Cholera toxin-pretreated membranes. The additive effects of cholera toxin with GTP and epinephrine and the lack of a cholera toxin response in the presence of Gpp(NH)p and fluoride prompted us to examine the responsiveness of adenylate cyclase in hepatocyte membranes that had been modified by cholera toxin-catalyzed ADP-ribosylation. Intact hepatocytes, rather than membranes, were practivated with cholera toxin because the toxin effect was more extensive in whole cells than in membrane preparations (see above). Accordingly, intact cells in monolayer were incubated with cholera toxin for 3 h, membranes prepared, and adenylate cyclase activity measured in response to various agents.

There was no effect of epinephrine on adenylate cyclase activity in toxin-activated membranes unless GTP was also present (Table III). The effect of GTP was additive with that of epinephrine in both untreated and cholera toxin-activated membranes. When toxin-activated membranes were exposed to Gpp(NH)p or fluoride, the adenylate cyclase activity was the same as that in untreated membranes. When cholera toxin-activated membranes were incubated with fluoride in the presence of GTP, there was a slight, but significant, increase in adenylate cyclase activity, suggesting that GTP may overcome somewhat the complete inhibition by fluoride of the cholera toxin response, Similarly, when toxin-activated membranes were incubated with epinephrine plus fluoride, the response was the same as that observed in control membranes exposed only to fluoride, and there was no effect of GTP.

To extend further these observations, cholera toxin-activated adenylate cyclase was compared with fluoride-pretreated and Gpp(NH)p-pretreated forms of the enzyme for their ability to respond to various agents (Table IV). Pretreatment conditions were chosen to activate the enzyme maximally. When

TABLE IV

EFFECTS OF PREACTIVATION OF HEPATOCYTE ADENYLATE CYCLASE WITH Gpp(NH)p OR NaF ON SUBSEQUENT ACTIVITIES

Membranes were pretreated in buffer (control), 100 μM Gpp(NH)p or 10 mM NaF for 10 min at 37°C, washed once, resuspensed in 1 mM $NaHCO_3$, and assayed for adenylate activity in the presence of the additions shown. The values shown are the means ± S.D. for six determinations.

Additions	Adenylate cyclase activity (pmol cyclic AMP/10 min per mg protein)		
	Control	Gpp(NH)p	NaF
Basal	102 ± 42	1158 ± 117	1006 ± 109
Cholera toxin (10 μg/ml)	157 ± 13	1389 ± 127	1328 ± 131
Epinephrine (5 μM)	171 ± 32	1262 ± 139	1408 ± 81
NaF (10 mM)	357 ± 63	1193 ± 93	3752 ± 311
GTP (100 μM)	159 ± 26	1262 ± 69	1026 ± 40
Gpp(NH)p (10 μM)	431 ± 37	1379 ± 151	2645 ± 272

membranes were pretreated with fluoride and subsequently assayed for adenylate cyclase activity, such membranes still responded to all agents tested, with the exception of GTP, although the magnitudes of stimulation by other agents were less than their effects on the enzyme of untreated membranes. In contrast, membranes pretreated with Gpp(NH)p were completely unresponsive to any other agent, while the adenylate cyclase of cholera toxin-treated membranes was responsive only to GTP and epinephrine (Table III).

Discussion

We have previously reported that cholera toxin ADP-ribosylates a 55 000 dalton membrane protein of RL-PR-C hepatocytes [21,29]. The goals of the present study were 2-fold: (1) to determine how such modification by cholera toxin affects the ability of hepatocyte adenylate cyclase to respond to catecholamine hormones, fluoride and guanine nucleotides; and (2) to investigate how cholera toxin-activated adenylate cyclase resembles cyclase that has been 'permanently' activated by fluoride or Gpp(NH)p.

It has been well-documented that the requirement for GTP in the activation of adenylate cyclase by hormones [30,31] and cholera toxin [32,33] is absolute. It is currently thought that cholera toxin exerts its major effect on the GTP regulatory processes of adenylate cyclase [14—16,32] perhaps by ADP-ribosylating a guanine nucleotide regulatory component associated with the enzyme complex [18—21]. The consequences of cholera toxin modification are complex. It has been suggested that such modification prolongs occupation of the guanine nucleotide regulatory component by GTP [15,16]. The enhancement of epinephrine responsiveness in toxin-treated hepatocytes (Fig. 1) would support this hypothesis, although we and others [14,32] have been unable to demonstrate an effect of cholera toxin on GTPase activity. The enhanced adenylase cyclase response to the non-hydrolyzable analogue of GTP, Gpp(NH)p (Table II), would also support the idea that hydrolysis of GTP is involved in the regulation of adenylate cyclase activity [34]. However, the

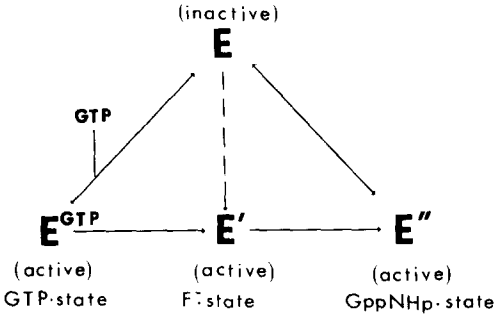

Fig. 2. Model of regulation of hepatic adenylate cyclase, GTP converts the inactive enzyme, E to a form, E^{GTP} which is responsive to hormones and may be stabilized by cholera toxin. Fluoride and Gpp(NH)p convert E^{GTP} to E′ and E″ respectively. Cholera toxin and hormones cannot convert E′ to E″ to E^{GTP} unless large concentrations of GTP are present.

effect of cholera toxin on the regulation of adenylate cyclase by guanine nucleotides seems more complex.

If cholera toxin merely prevents hydrolysis of GTP at the guanine nucleotide regulatory site [15,16], then the activation of adenylate cyclase by GTP in cholera toxin-treated membranes should resemble the activation by Gpp(NH)p in the absence of the toxin. Clearly, the data in Table III show this not to be the case. Similar observations have been made in other systems [32,35,36]. Flores and Sharp [36] concluded that responsiveness to cholera toxin and Gpp(NH)p could not be expressed simultaneously and that activation by these two ligands was competitive, since both ligands interacted with the same site. Additionally, Lin et al. [32] found that cholera toxin decreased the affinity of guanine nucleotides for the processes involved in the activation of adenylate cyclase and in the regulation of the binding of glucagon to its receptor. These authors proposed that cholera toxin may alter the conformation of the guanine nucleotide regulatory component associated with adenylate cyclase. The present findings would support the proposal that cholera toxin and Gpp(NH)p are competitive and that Gpp(NH)p responsiveness predominates over that to cholera toxin since responsiveness to Gpp(NH)p is the same in both control and cholera toxin-treated membranes.

Actually, Gpp(NH)p seems capable of inducing a state of adenylate cyclase activity that is different from the state stabilized by cholera toxin. If GTP and Gpp(NH)p compete for the same site [37], and the affinity of this site for GTP is higher than for Gpp(NH)p (Table II), then Gpp(NH)p-activated membranes should respond to GTP as do untreated membranes. Clearly, this was not the case (Table IV). Gpp(NH)p seems to stabilize a state of adenylate cyclase that exhibits Gpp(NH)p responsiveness regardless of the presence of other activators of the enzyme. It has been proposed that Gpp(NH)p is only slowly dissociable from the guanine nucleotide regulatory site [37], but in the presence of GTP, significant displacement of Gpp(NH)p should occur in 10 min [37].

The mechanism of fluoride activation of adenylate cyclase is poorly understood, but it is thought to involve the guanine nucleotide regulatory protein [38,39]. Hormones [30,31] and cholera toxin [32,33] appear to activate adenylate cyclase only if GTP is bound to the regulatory site, whereas fluoride

may activate the enzyme if this site is occupied by GDP [39] or is empty [38]. It has consistently been found that the fluoride response in many tissues is reduced or even absent if the membranes have been pretreated with cholera toxin and NAD^+ [22,36,40–43]. We can confirm this observation with RL-PR-C hepatocytes. When these cells were first exposed to cholera toxin, and their membranes then incubated with fluoride, there was no further response to the ion (Table III); the response to fluoride (or Gpp(NH)p was the same whether or not the cells were pre-exposed to the toxin. Thus, responsiveness to cholera toxin, fluoride and Gpp(NH)p cannot be expressed simultaneously. Further, when the adenylate cyclase of hepatocytes was exposed to various ligands, the response to Gpp(NH)p predominated over the response to cholera toxin and fluoride, and the response to fluoride predominated over that to cholera toxin and epinephrine.

All of the above observations are consistent with the following model for the regulation of adenylate cyclase activity (Fig. 2). According to this model, adenylate cyclase exists in at least four separate forms, the equilibrium between which depends on the presence of various activating ligands. When GTP occupies the nucleotide regulatory site, i.e., E^{GTP}, the enzyme is responsive to hormones. This form of the enzyme may be stabilized by cholera toxin, as proposed by Cassel and Selinger [16]. However, fluoride induces a state of the enzyme, E′, of which the responsiveness to other ligands is reduced, while Gpp(NH)p induces a state of the enzyme, E″, that no longer responds to any other agent. Such a model would explain why responsiveness to cholera toxin is exclusive of responsiveness to fluoride and Gpp(NH)p. Exposure of adenylate cyclase to cholera toxin and/or hormones in the presence of fluoride pulls the equilibrium in the direction of E′, while exposure of the enzyme to any other agent in the presence of Gpp(NH)p pulls the equilibrium in favor of E″, both conversions being irreversible unless excess GTP is present. Such a model might explain why high concentrations of GTP inhibit fluoride activation of adenylate cyclase [44] by converting E′ to E^{-GTP}, E^{-GTP} activity being less than E′. Most likely, these various forms of adenylate cyclase represent conformational states, as proposed by Rodbell et al. [45]. These authors hypothesize that the enzyme exists in different transitional states which have different kinetic properties at the active site, and that binding of ligands to the cyclase complex shifts the equilibrium between these various states. There is physical evidence that demonstrates that the conformation of the nucleotide regulatory unit of the adenylate cyclase complex is altered depending on the nature of the attached ligand [32,46,47]. It is also possible that E′ and E″ represent adenylate cyclase with GDP and GMP, respectively, bound to the nucleotide regulatory site. The alteration of the fluoride response in cholera toxin-treated membranes by GTP would support this conclusion.

The present study demonstrates that the regulation of hepatic adenylate cyclase is exceedingly complex. Clearly, cholera toxin-catalyzed ADP-ribosylation has some effect on guanine-nucleotide-regulated processes. However, hepatic adenylate cyclase activity is not solely regulated by GTP. Cholera toxin, fluoride and Gpp(NH)p are all capable of activating adenylate cyclase and each induces a separate, distinct enzyme state. Whether all of these effects are mediated through the same regulatory component or different components asso-

ciated with the adenylate cyclase complex must await purification and reconstitution of the resolved components of the enzyme.

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