INFLUENCE OF CHOLERA TOXIN ON THE REGULATION OF ADENYLATE CYCLASE BY GTP

Gary L. Johnson* and Henry R. Bourne+

Departments of Medicine*+, Pharmacology+,
and Cardiovascular Research Institute*+,
University of California, San Francisco,
San Francisco, California 94143

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SUMMARY: In the presence of NAD^+, cholera toxin activates adenylate cyclase in membranes of S49 mouse lymphoma cells. The following evidence supports the hypothesis that the toxin acts by inhibiting a specific GTPase associated with a guanyl nucleotide regulatory component of hormone-responsive cyclase: 1. GTP alone markedly stimulates cyclase activity in toxin-treated, but not in untreated membranes; 2. The poorly hydrolyzable GTP analog, guanosine 5'-(β,γ-imino) triphosphate (Gpp(NH)p), stimulates cyclase equally well in toxin-treated and untreated membranes; 3. Cyclase activation by isoproterenol plus GTP persists in toxin-treated membranes, but not in controls, after addition of propranolol; 4. GTP is a more potent competitive inhibitor of the irreversible activation of cyclase by Gpp(NH)p in toxin-treated than in untreated membranes.

INTRODUCTION: Cholera toxin stimulates synthesis of cyclic adenosine 3',5'-monophosphate (cAMP) and increases the sensitivity of adenylate cyclase to hormones in a wide variety of animal cells and tissues (1-3). In broken cell preparations, these effects of the toxin depend upon the presence of NAD^+ (4,5). The ubiquity and uniformity of cholera toxin's action in animal cells suggests that it acts on a component common to all hormone-responsive adenylate cyclase systems. Thus, understanding of the mechanism of cholera toxin's action should provide insight into the molecular basis of hormonal regulation of adenylate cyclase.

One component of all hormone-responsive adenylate cyclase systems so far studied is a guanyl nucleotide regulatory site. Cassel and Selinger have recently demonstrated (6,7) an isoproterenol-stimulated GTPase activity that appears to be associated with this nucleotide regulatory site in turkey erythrocyte membranes. The subsequent observation (8) that this hormone-stimulated GTPase activity is inhibited

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by cholera toxin led to the postulate that toxin stimulates cAMP synthesis by blocking a specific GTPase activity that normally functions to turn off adenylate cyclase. We have tested this hypothesis by examining the regulation of adenylate cyclase by guanyl nucleotides in mouse S49 lymphoma membranes treated with cholera toxin and NAD^+. Our results support the hypothesis.

METHODS: Cultured S49 mouse lymphoma cells (9) were grown in Dulbecco's modified Eagle's medium with 10% heat inactivated horse serum (10). Cholera toxin was obtained from Schwarz-Mann. [^{3}H] cAMP was purchased from New England Nuclear Corporation. (±)-Isoproterenol, (±)-propranolol, NAD^+, and GTP were from Sigma. Gpp(NH)p was purchased from ICN.

Plasma membrane purification was accomplished by the method of Ross et al (11) with modification. S49 cells were harvested by low speed centrifugation, washed once with Puck's phosphate buffered saline, and resuspended at a density of 10^7/ml in 150 mm NaCl, 20 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.4 at 4°. Cells were disrupted by nitrogen cavitation (500 psi, 20 min at 4°) using a Parr nitrogen disruption apparatus (Parr Instrument Co., Moline, Ill.). The 'bombate" was centrifuged at 750xg for 5 min and the supernatant then centrifuged at 43,000xg for 20 min. The pellet was resuspended using a Dounce homogenizer in 10% sucrose (w/w), 20 mM NaHepes, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 8.0 and layered over a stepwise gradient of 40, 30, 20% sucrose (w/w) in the same buffer and centrifuged in an SW27.1 rotor at 25,000 rpm for 80 min. The membranes at the 30-40% and 20-30% interfaces were collected and combined, diluted with the same buffer without sucrose and centrifuged at 43,000xg for 20 min. Using a Dounce homogenizer, the pellet was resuspended in the same buffer (plus 10% glycerol), frozen in dry ice-ethanol, and stored at -70°. Hormone and cholera toxin responsiveness was stable for at least 3 months.

Adenylate cyclase was measured essentially as described previously (12). ATP (1 mM) was used as substrate in a medium of 50 mM NaHepes, 4 mM $MgCl_2$, 0.2 mM 1-methyl 3-isobutyl-xanthine, 5 mM 2-mercaptoethanol, 0.2 mg/ml bovine serum albumin, pH 8.0 in a final volume of 100 µl. Cyclic AMP was measured by the competitive protein binding method (13). Incubations were initiated by addition of membranes (approximately 10 µg) and continued for 10 min at 30° and terminated by addition of 50 µl 150 mM acetic acid. Sodium flouride stimulated cyclase activity under these conditions was linear with time and protein concentration.

Cholera toxin was preactivated (14,15) with 20 mM dithiothreitol for 10 min at 30°. Membranes were then incubated for 5 min with the appropriate concentrations of cholera toxin and NAD^+ and immediately transferred to the adenylate cyclase reaction mixture.

RESULTS AND DISCUSSION: Hormone-responsive adenylate cyclase activity of highly purified plasma membranes of S49 cells has an absolute requirement for the presence of a guanine nucleotide such as GTP or Gpp(NH)p (11). In the absence of hormone, however, GTP is virtually inactive, while the

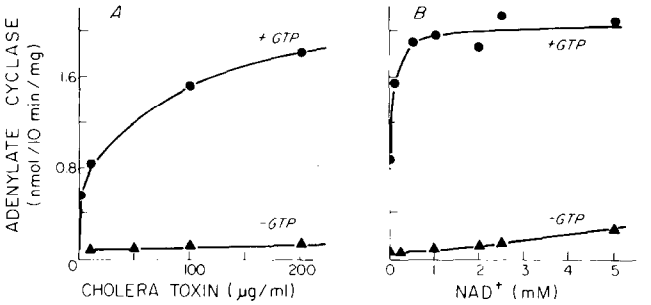

Fig. 1. Activation of adenylate cyclase by cholera toxin and NAD^+. Membranes were exposed to various concentrations of (A) cholera toxin (plus 2.5 mM NAD^+) or (B) NAD^+ (plus 100 μg/ml cholera toxin). The membranes were then added to the adenylate cyclase reaction mixture with or without 100 μM GTP. Values represent the mean of duplicate samples which differed by less than 10%.

GTP analog Gpp(NH)p is still capable of activating adenylate cyclase. This is apparently due to Gpp(NH)p's resistance to hydrolysis of the terminal phosphate linkage (16). In the presence of NAD^+, cholera toxin activates adenylate cyclase, but the requirement of GTP for enzymatic activity persists (Fig. 1A). This is in contrast to activation by NaF which occurs in the presence or absence of GTP (data not shown). NAD^+ increased the activation of adenylate cyclase in a concentration-dependent fashion, although the requirement for exogenously added NAD^+ was not absolute (Fig. 1B). In several experiments with different membrane preparations, cyclase activation by cholera toxin (10 μg/ml) without added NAD^+ varied between 2- and 12-fold, while 20- to 40-fold increases were observed when toxin was used with 2.5 mM NAD^+. Activation in the absence of added NAD^+ is probably due to varying amounts of residual endogenous NAD^+ bound to the membranes (4).

Fig. 2 compares the response of cyclase activity in control and toxin-activated membranes to various concentrations of GTP and Gpp(NH)p, in the presence or absence of 10 μM isoproterenol. GTP alone stimulates cyclase activity in toxin-treated membranes, but not in controls. At all concentrations of GTP, the cyclase response to isoproterenol is

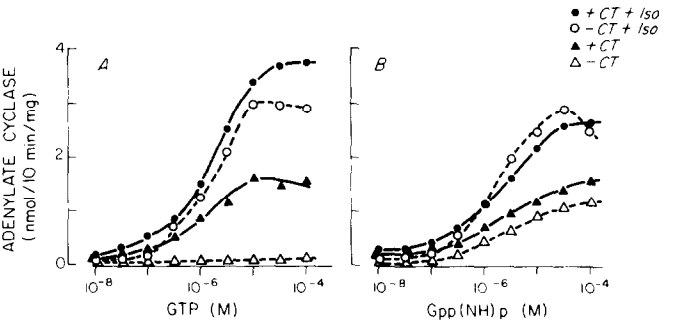

Fig. 2. Responsiveness of adenylate cyclase in control and cholera toxin-treated membranes to various concentrations of GTP and Gpp(NH)p in the presence or absence of isoproterenol.

Membranes were incubated in the presence or absence of 10 µg/ml cholera toxin and 2.5 mM NAD^+ and then transferred to the adenylate cyclase reaction mixture containing various concentrations of (A) GTP or (B) Gpp(NH)p in the presence or absence of 10 µM (±)-isoproterenol. Values represent the mean of duplicate samples which differed by less than 10%.

consistently greater in toxin-treated than in control membranes. In contrast, no consistent difference can be seen between toxin-treated and control membranes in response to Gpp(NH)p, with or without isoproterenol.

The fact that toxin enhances the stimulatory effects of GTP, but not that of its poorly hydrolyzable analog, suggests that the toxin may inhibit hydrolysis of GTP. If so, GTP-dependent cyclase activation in toxin-treated membranes should resemble Gpp(NH)p activation of cyclase in control membranes, in that both should persist with time after the initial stimulus is removed. To test this prediction, we exposed membranes to isoproterenol plus GTP or Gpp(NH)p for 5 min and then added a high concentration of the β-antagonist, propranolol (Fig. 3). In control membranes exposed to isoproterenol plus GTP, addition of propranolol immediately abolishes cyclase activation, as would be expected if GTP at the nucleotide regulatory site is rapidly hydrolyzed. In toxin-treated membranes, however, activation by isoproterenol plus GTP persists almost unchanged for at least 5 min after addition of propranolol (Fig. 3A), as predicted. This persistent activation by GTP in toxin-

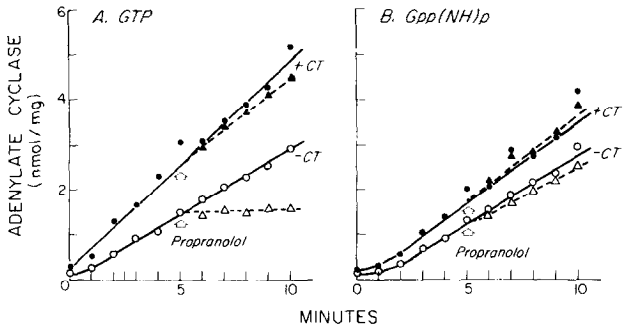

Fig. 3. Effect of cholera toxin on the persistance of isoproterenol-stimulated adenylate cyclase activity in the presence of GTP or Gpp(NH)p. Membranes were incubated in the presence or absence of 10 µg/ml cholera toxin and 2.5 mM NAD^+ and then transferred to a 4 ml adenylate cyclase reaction mixture containing (A) 100 µM GTP or (B) 100 µM Gpp(NH)p in the presence of 10 µM (±)-isoproterenol. At the appropriate intervals duplicate 100 µl aliquots were withdrawn and added to the stop solution. At 5 min 1.2 ml samples were withdrawn to which (±)-propranolol was added to give a final concentration of 50 µM and duplicate 100 µl aliquots withdrawn at appropriate intervals. Values represent the mean of duplicate samples which differed by less than 10%.

treated membranes closely resembles that caused by Gpp(NH)p (Fig. 3B), which has been reported in other systems (8,16,17). The Gpp(NH)p effect persists after addition of propranolol in both toxin-treated and control membranes (Fig. 3B).

Unlike the effect of Gpp(NH)p, GTP's cyclase stimulation does not withstand dilution and washing of membranes, even in toxin-treated membranes, perhaps because GTP hydrolysis is only partially inhibited by the toxin. This fact allows us, however, to ask whether the two guanyl nucleotides occupy the same regulatory site, by assessing the ability of GTP to compete for that site with Gpp(NH)p, and thus to prevent the latter's irreversible activation of cyclase (Fig. 4). GTP is 10-fold more potent as a competitive blocker of Gpp(NH)p's activation of cyclase in toxin-treated than in control membranes (Fig. 4). Under the same conditions, GDP also prevents activation by Gpp(NH)p, with a potency (apparent K_i = 1.0 µM) that is similar in toxin-treated and control membranes (not shown). These results strongly suggest that toxin treat-

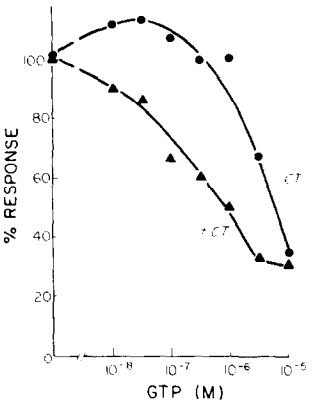

Fig. 4. Competition of GTP and Gpp(NH)p for the guanyl nucleotide regulatory site of cholera toxin-treated and control membranes.

Membranes were incubated in the presence or absence of 10 µg/ml cholera toxin and 2.5 mM NAD^+ and then transferred to the adenylate cyclase reaction mixture containing 10 µM Gpp(NH)p in the presence of various concentrations of GTP (ATP omitted). Following a 10 min incubation the membranes were diluted 20-fold and centrifuged at 27,000xg for 15 min. The membranes were resuspended with 5 strokes of a Dounce homogenizer and transferred to the adenylate cyclase reaction mixture. Adenylate cyclase activity in the presence of 10 µM Gpp(NH)p and no GTP was 2.1 nmoles/10 min/mg with toxin-treated membranes and 1.2 nmoles/10 min/mg with control membranes. Values represent the mean of duplicate samples which differed by less than 10%.

ment prolongs the occupancy of a guanyl nucleotide regulatory site by GTP, as would be expected if the toxin partially inhibits GTP hydrolysis.

Our findings, in a mammalian cell line, strongly support the conclusion drawn from experiments with avian erythrocytes (8) that cholera toxin activates adenylate cyclase by inhibiting a GTPase activity associated with hormone-sensitive cyclase. Furthermore, the GTP-Gpp(NH)p competition experiment (Fig. 4) suggests that this GTPase activity occurs at the same (or a closely related) site as that occupied by Gpp(NH)p to activate the cyclase.

In preliminary experiments, we have not been able reproducibly to detect a hormone-stimulated or cholera toxin-inhibited GTPase activity in S49 membranes, as a direct confirmation of the turkey erythrocyte experiments. If such measurements can be made reproducible, an activity of the guanyl nucleotide regulatory site can be investigated in clonally selected

variant S49 cells with defects in hormonal stimulation of cAMP synthesis (18,19). Such a genetic approach may help in understanding the mysterious "coupling" mechanism that links hormone receptors to adenylate cyclase.

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