INHIBITION OF GUANYLATE CYCLASE AND CYCLIC GMP PHOSPHODIESTERASE BY CHOLERA TOXIN *

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SUMMARY: Results of cholera toxin exposure in rabbit small intestinal epithelial cells, following 4 to 6 hours of incubation, indicate that there is simultaneous dose-dependent activation of adenylate cyclase and deactivation of guanylate cyclase. In addition, cyclic GMP phosphodiesterase activity is repressed. These data indicate that cholera toxin interacts with a binding site of dissociation constant K_d=3.8±1.3 X 10^{-9}M to produce multiple coordinated events in the cells.

INTRODUCTION: Cholera toxin has recently been recognized as a potent activator of adenylate cyclase (EC 4.6.1.1) in small intestinal epithelial cells (1) (2), and subsequently, in virtually every cell type of mammalian origin tested (3). Elevation of intracellular levels of cyclic AMP are achieved by cholera toxin as a function of an enhanced rate of synthesis of cyclic AMP by adenylate cyclase without any apparent change in the rate of hydrolysis by phosphodiesterase (EC 3.1.4.-) (2).

To date, the only other naturally occurring 3'5' cyclic nucleotide, cyclic GMP, has not been considered to be influenced by cholera toxin. When steady-state tissue levels of cyclic GMP were measured in dog jejunal mucosa (4) and rat thymocytes (5) between 5 minutes and 3 hours after exposure to cholera toxin, concentrations of cyclic GMP were unchanged.

Yet, a considerable body of evidence has accumulated in support of the "Yin-Yang" hypothesis of Goldberg (6) (7), that a reciprocal biological

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dualism exists between cyclic AMP and cyclic GMP, so that the ratio of the two nucleotides within cells might thus regulate many cellular functions. For this reason it seemed of considerable interest to determine whether cholera toxin influenced those enzymes responsible for maintaining the steady state concentrations of cyclic GMP. Directly demonstrated effects on enzyme function are frequently more revealing of the nature of processes occuring than are studies of whole cell levels of enzyme products. In the present study, the effect of cholera toxin on guanylate cyclase activity and rates of hydrolysis by cyclic GMP phosphodiesterase were examined in rabbit small intestinal epithelial cells, at periods of 4-6 hours subsequent to toxin exposure, a time when adenylate cyclase activation has normally reached its maximum (8). The data indicate reductions in guanylate cyclase activity directly paralleling increases in adenylate cyclase activity at similar toxin doses. Cyclic GMP phosphodiesterase is also inhibited by cholera toxin exposure.

The similar dose-response behavior between the activation of adenylate cyclase and inhibition of guanylate cyclase reveals that cholera toxin is interacting with a receptor which has a pervasive role in regulating multiple coordinated events within a cell.

MATERIALS AND METHODS:

Toxin Exposure: Purified cholera toxin was obtained from the NIH Cholera Advisory Committee, Bethesda, Maryland. This material had been prepared by the method of Finkelstein and LoSpalluto (9). Stock solutions of cholera toxin were serially diluted in phosphate-buffered saline (0.85% NaCl, 0.15 M Phosphate, pH 7.5). Laparotomy was performed on 2-3 Kg male New Zealand rabbits (Scientific Small Animals, Arlington Heights, Ill.) under intravenous pentobarbital anaesthesia. Segments of the distal ileum were flushed with saline and ligated to construct 4 cm closed loops interfaced with 2 cm intermediate spacing loops at both ends. Injections of 2 ml of cholera toxin solutions of varying concentrations were made into the lumen of the 4 cm loops with 25 gauge needles. Alternate injections of high and low toxin concentrations were made to randomize toxin concentration along the ileum. Animals were sacrificed after 4-6 hour incubation periods by overdose of pentobarbital.

Tissue Preparation: Ileal loops were immediately excised from sacrificed animals, slit open, blotted and quickly rinsed three times in 75mM Tris buffer containing 1mM dithiothreitol, pH 7.5, at 4°. Loops were placed epithelial cell side up on filter paper in a bed of ice; epithelial cells were scraped and homogenized in 2 volumes of the previous buffer by 10 strokes in a chilled glass Tenbroeck tissue homogenizer. Homogenates were immediately aliquotted to prepared assay mixtures held at 4°C. Time between sacrifice of animals and completion of assays was held to one hour.

Protein Determinations: Protein content of each homogenate was determined

in quadruplicate by the method of Geiger and Bessman (10). This method is a modification of the procedure by Lowry et al (11) in which hydrogen peroxide is used to destroy all dithiothreitol present in the homogenates. Dithiothreitol has been shown to artifactually elevate protein values by standard Lowry determinations (10). Bovine Serum Albumin (Sigma) was used as a protein standard.

Adenylate Cyclase Assay: Adenylate cyclase was assayed as previously reported by this laboratory (12). The phosphodiesterase inhibitor, aminophylline, previously used, was replaced in this assay by 1mM MIX (1-methyl, 3-isobutyl xanthine).

Guanylate Cyclase Activity: Guanylate cyclase was assayed in 30 mM Tris containing 10 mM $MnCl_2$, 5 mM KCl, 0.2 mM cyclic GMP and 1mM MIX at pH 7.5. Reactions were started by the addition of 1 mM GTP, (Sigma) containing [^{32}P]-GTP (New England Nuclear) for a final specific activity at 90-110 cpm/pmol. Final assay volumes were 50 µl; assay tubes were incubated at 37° for 5 min and stopped by boiling for 3 min. On one occasion reactions were stopped by the addition of 50 mM sodium acetate buffer, pH 4, and 1 mM cyclic GMP (13), to prevent non-enzymatic conversion of [^{32}P]-GTP to cyclic [^{32}P]-GMP during boiling. Following centrifugation, aliquots of 25 µl from each assay mixture were removed and quantitatively spotted on cellulose-polyethylenimine sheets (Brinkman) for chromatography by the method of Keirns et al (14). [^{32}P]-cyclic GMP spots were then cut out and counted by liquid scintillation spectrometry. The identity of the [^{32}P]-cyclic GMP spot was confirmed by hydrolysis with cyclic 3'5'-nucleotide phosphodiesterase (Sigma).

Assay of Cyclic GMP Phosphodiesterase: The method of Thompson and Appleman (15) was used. [^{3}H]-cyclic-3'5'-GMP was obtained from New England Nuclear, cobra venom and 5'- nucleotidase were obtained from Sigma. Results are expressed as pmole cyclic GMP hydrolyzed per minute per mg homogenate protein.

Statistics: For each experimental point triplicate determinations of each velocity assay were preformed, while protein was determined in quadruplicate. Standard deviations of the mean (σm) for each final velocity and protein value were combined in the equation, $R = \sqrt{\left(\frac{\sigma_{m,p}}{[p]}\right)^2 + \left(\frac{\sigma_{m,v}}{v}\right)^2}$ in which R is the relative error of the final result expressed in pmol/min/mg homogenate protein, and p and v denote protein (mg) and velocity (pmol/min), respectively. Error bars depicted were calculated in this way. In addition, each group of experiments were performed at 4, 5 and 6 hr intervals subsequent to toxin exposure, with identical results.

RESULTS AND DISCUSSION: Results of guanylate cyclase determinations obtained at varying incubation concentrations of cholera toxin are shown in Fig. 1. Also depicted is a theoretical binding curve constructed according to a standard binding analysis, $v = \frac{V_{max}}{1 + [T] / K_d}$ where [T] represents toxin concentration, and values of the apparent dissociation constant K_d = 0.37 µg/ml and the maximal velocity V_{max} = 10.5 pmol/min/mg, have been obtained from a linear plot of 1/v vs. [T][1]. For comparison Fig. 1 also shows assays of

[1] The data given in Fig. 1 are for whole-cell homogenates. When cells were fractionated in a preliminary experiment, reduction in guanylate cyclase activity appeared slightly more marked in the soluble cell fraction than in the particulate fraction (16).

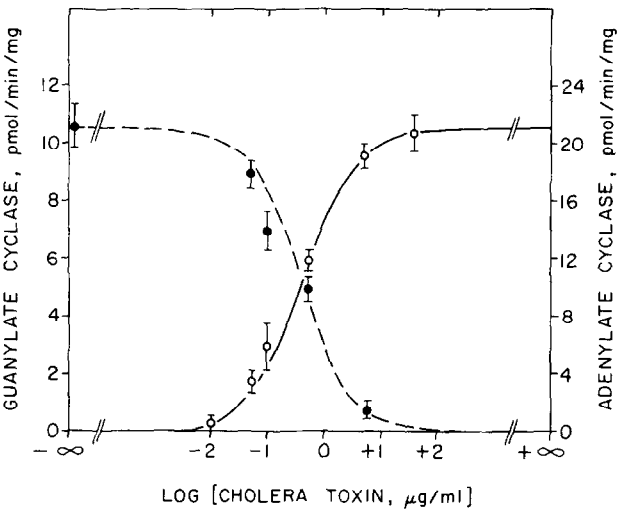

Figure 1. Changes in guanylate (•) and adenylate (o) cyclase activities in rabbit intestinal epithelial cells subsequent to 5 hr incubation with varying concentrations of cholera toxin. Experimental points are contrasted with theoretical binding curves: guanylate cyclase (- - -), K_d=0.37 μg/ml toxin, V_{max}= 10.5 pmol/min/mg protein; adenylate cyclase (————), K_d=0.42 μg/ml toxin, V_{max}= 21 pmol/min/mg protein. Both assays were performed using whole cell homogenates with substrate at 10-fold excess over Km, in 30mM Tris, pH 7.5, 1mM MIX, 37°. (For metal ion additions, see Methods.) Identical results were obtained for incubation times of 4 and 6 hrs.

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adenylate cyclase velocities at varying incubation concentrations of cholera toxin which were performed after the same time intervals (4 - 6 hrs) as the guanylate cyclase determinations. The experimental data are compared to a theoretical binding curve constructed with K_d=0.42μg/ml and V_{max}= 21 pmol/min/mg. The results delineate the profile of the well established activation of adenylate cyclase by cholera toxin.

The activity of phosphodiesterase(s) catalyzing the hydrolysis of cyclic GMP was also reduced in a dose-dependent fashion subsequent to cholera toxin exposure (Fig. 2). Values of V_{max}= 3300 pmol/min/mg and K_d=0.17 μg/ml were obtained from a linear replot (inset, Fig.2), and these values were used to construct the theoretical curve shown. Approximately one out of every 15 intestinal loops assayed failed to show a depression in cyclic GMP phosphodiesterase activity upon cholera toxin exposure, regardless of toxin concen-

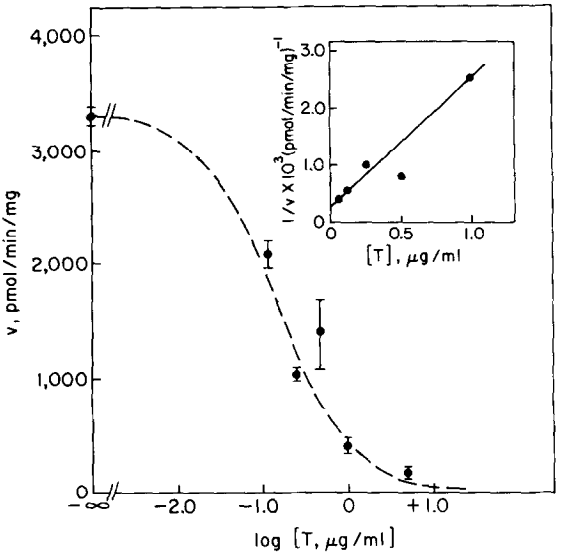

Figure 2. Changes in cyclic GMP phosphodiesterase activity levels (●) in rabbit intestinal epithelial cells subsequent to 5 hr incubation with varying concentrations of cholera toxin. The inset shows a linear double-reciprocal binding determination yielding values of K_d=0.17 μg/ml toxin and V_{max}=3300 pmol/min/mg protein. Experimental points are contrasted with a theoretical binding curve (- - -) constructed from these V_{max} and K_d values. Assay of whole-cell homogenates were performed in 40 mM Tris, pH 8.0 with 2 mM mercaptoethanol, 5mM Mg^{++} and 0.1mM [^{3}H]-cyclic GMP, at 30°. Identical results were obtained for incubation times of 4 and 6 hrs.

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tration. As a result, each of three groups of experiments contains a single high experimental point of high standard deviation. The meaning of this finding is unclear, but it was shown experimentally to be unrelated to the position of the loop in the ileum.

Results presented above clearly indicate that the cellular activities related to cyclic GMP metabolism in rabbit intestinal epithelial cells are strikingly modified subsequent to cholera toxin exposure. Values of the apparent dissociation constant representing the interaction of the toxin molecule (M.W. 84,000) with the receptors mediating all three observed processes are very similar: adenylate cyclase activation, K_d= 5.0 X 10^{-9}M; guanylate cyclase inhibition, K_d= 4.4 X 10^{-9}M; cyclic GMP phosphodiesterase repression, K_d= 2.1 X 10^{-9}M.

The above findings are in contrast to the previous findings of Schaefer et al (4) and Boyle et al (5) that the cyclic GMP system does not appear to respond to cholera toxin. Both of these reports were based on the lack of change in whole cell levels of cyclic GMP, assayed at intervals from 5 min to 3 hrs following exposure to cholera toxin. Two features of the current experiments may account for this difference: (a) our results were obtained over a 4 to 6 hr period following initial toxin contact, a time at which adenylate cyclase activation is known to have been completed; (b) the simultaneous dose-dependent reductions of both guanylate cyclase and cyclic GMP phosphodiesterase which we found could compensate and account for apparent unchanging steady state whole cell levels of cyclic GMP at any given time.

From the mathematical analysis of the dose-dependent behavior of guanylate cyclase, adenylate cyclase, and cyclic GMP phosphodiesterase, it appears clear that all three result from a saturable binding by cholera toxin at a class of sites of average affinity represented by $K_d = 3.8 \pm 1.3 \times 10^{-9}$M. This value corresponds closely to the reported dissociation constants (4.6×10^{-10}M and 1.1×10^{-9}M) obtained for the interaction of cholera toxin with fat cell and liver membranes by Cuatrecasas (17).

The biological meaning of the inhibition of both the production and the destruction of cyclic GMP in response to a single agent is subtle. Since with this double inhibition, cyclic GMP metabolism would be virtually paralyzed in the face of rising cyclic AMP levels, these data would tend to imply that the ratio between cyclic GMP and cyclic AMP levels constitutes the biologically meaningful signal. In summary, the binding of cholera toxin to its receptor appears to be an event of profound importance to the intracellular balance between cyclic nucleotides which may play such a vital role in cellular regulatory machinery.

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