J. Membrane Biol. 24, 107–129 (1975)
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Mechanism of Activation of Adenylate Cyclase by *Vibrio cholerae* Enterotoxin

Relations to the Mode of Activation by Hormones

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Received 8 April 1975; revised 9 June 1975

Summary. The influence of *Vibrio cholerae* enterotoxin (choleragen) on the response of adenylate cyclase to hormones and GTP, and on the binding of ^{125}I-labeled glucagon to membranes, has been examined primarily in rat adipocytes, but also in guinea pig ileal mucosa and rat liver. Incubation of fat cells with choleragen converts adenylate cyclase to a GTP-responsive state; (−)-isoproterenol has a similar effect when added directly to membranes. Choleragen also increases by two- to fivefold the apparent affinity of (−)-isoproterenol, ACTH, glucagon, and vasoactive intestinal polypeptide for the activation of adenylate cyclase. This effect on vasoactive intestinal polypeptide action is also seen with the enzyme of guinea pig ileal mucosa; the toxin-induced sensitivity to VIP may be relevant in the pathogenesis of cholera diarrhea. The apparent affinity of binding of ^{125}I-labeled glucagon is increased about 1.5- to twofold in choleragen-treated liver and fat cell membranes. The effects of choleragen on the response of adenylate cyclase to hormones are independent of protein synthesis, and they are not simply a consequence of protracted stimulation of the enzyme *in vivo* or during preparation of the membranes. Activation of cyclase in rat erythrocytes by choleragen is not impaired by agents which disrupt microtubules or microfilaments, and it is still observed in cultured fibroblasts after completely suppressing protein synthesis with diphtheria toxin. Choleragen does not interact directly with hormone receptor sites. Simple occupation of the choleragen binding sites with the analog, choleragenoid, does not lead to any of the biological effects of the toxin.

Cholera enterotoxin, an oligomeric protein secreted by *Vibrio cholerae,* stimulates ubiquitously adenylate cyclase activity in mammalian tissues (Finkelstein, 1973; Sharp, 1973). The initial event in the action of cholera toxin (choleragen) involves high affinity binding to cell surface receptor sites (Cuatrecasas, 1973*a–c*; Boyle & Gardner, 1974; Hollenberg *et al.*, 1974; Holmgren *et al.*, 1974; Walker *et al.*, 1974) which are believed to be G_{M1} monosialogangliosides (Cuatrecasas, 1973*a–c*; Holmgren *et al.*, 1973*a*, *b*; King & van Heyningen, 1973; Pierce, 1973; Hollenberg *et al.*, 1974; Holmgren *et al.*, 1974; van Heyningen, 1974). This glycolipid competes very effectively for the binding of ^{125}I-labeled cholera toxin

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to membranes (Cuatrecasas, 1973*a,b*) and it blocks the biological effects of the toxin (Cuatrecasas, 1973*a–c*; Holmgren *et al.*, 1973*a, b*; King & van Heyningen, 1973; Pierce, 1973). Moreover, exogenous ganglioside can incorporate spontaneously into cell membranes, resulting in enhanced binding of choleragen as well as in an increase in the sensitivity of lipolysis and adenylate cyclase to the toxin (Cuatrecasas, 1973*b*)[1].

The availability of a protein which binds selectively to a chemically defined cell surface receptor, and which activates adenylate cyclase specifically, suggests special ways of studying the mechanisms of hormonal modulation of this enzyme. Stimulation of adenylate cyclase activity of toad erythrocytes by choleragen involves changes in the kinetic and regulatory properties of this enzyme (Bennett & Cuatrecasas, 1974; 1975*a*). The alterations induced by choleragen resemble closely the effects of catecholamine hormones in these cells (Bennett & Cuatrecasas, 1974; 1975*a*). Choleragen increases the maximal response of adenylate cyclase of toad erythrocyte plasma membranes to catecholamines, and it increases the apparent affinity of these hormones for activation of the enzyme from rat erythrocyte membranes (Bennett & Cuatrecasas, 1974; 1975*a*). Increased sensitivity of adenylate cyclase activity to catecholamines has also been reported for turkey erythrocytes (Field, 1974), and rat epididymal fat cells (Hewlett *et al.*, 1974). Choleragen also increases the apparent affinity of rat liver adenylate cyclase for activation by glucagon, and it decreases the rate of spontaneous dissociation of ^{125}I-labeled glucagon from rat liver membranes (Bennett *et al.*, 1975).

The present studies examine the influence of choleragen on the response of adenylate cyclase activity of rat fat cells to GTP and to a variety of lipolytic hormones, extend some of these findings to the guinea pig ileal mucosa, and explore possible interactions of choleragen with receptors for hormones.

Materials and Methods

Cholera toxin (lot #1071) and choleragenoid (lot #G0673), purified by the method of Finkelstein and LoSpalluto (1970), were obtained from Dr. C.E. Miller, SEATO Cholera Research Program. 5′ATP, 5′GTP, phosphoenolpyruvate, (−)-epinephrine bitartrate, (−)-isoproterenol HCl, propranolol HCl, phentolamine HCl, neutral alumina, aminophylline and Trizma base were purchased from Sigma; 2′3′-isopropylidene adenosine was from Aldrich, pyruvate kinase and myokinase from Boehringer, staphylococcal nuclease (6,000 units/mg) and crude collagenase (Type I, 232 units/mg) from Worthington, bovine serum albumin (Fraction V) from Armour, and crystalline glucagon and ACTH were from Eli Lilly. [^{32}P]-Orthophosphoric acid (carrier free) in 0.02 N HCl and [^{3}H]-cAMP (22 Ci/mmole) were obtained from New England Nuclear, and carrier-free Na^{125}I was from Union Carbide. Pure vasoactive intestinal peptide (VIP) was generously contributed by Dr. Sami Said. Diph-

1 *Also,* B. Beckman, E. O'Keefe and P. Cuatrecasas (*manuscript in preparation*).

theria toxin was purified to homogeneity (on SDS disc gel electrophoresis) from crude material (Wyeth Laboratories, lot 12970) essentially by the procedures described by Goor and Pappenheimer (1967).

[α-^{32}P]-ATP (10–20 Ci/mmole) was synthesized and purified as described elsewhere (Bennett & Cuatrecasas, 1975*a*). ^{125}I-Labeled cholera toxin (Cuatrecasas, 1973*a*) and glucagon (Desbuquois *et al.*, 1974) were prepared as reported previously. Before iodination and biological studies, choleragen and choleragenoid were chromatographed on G-75 Sephadex columns (1 cm × 20 cm) equilibrated with 0.25 M Na_2PO_4, pH 7.4.

Isolated fat cells were prepared from epididymal fat pads of Sprague-Dawley male rats (80 to 140 g) essentially by the method of Rodbell (1964). Fat cell "membranes" (whole particulate fraction) were isolated by homogenizing adipocytes in ice-cold Tris-HCl (50 mM, pH 7.7) with a Brinkman Polytron (20 sec at a setting of 2.8) followed by centrifugation at 0 °C for 20 min at 40,000 × *g*. The membrane pellet was resuspended in Tris-HCl (50 mM, pH 8.0) at a concentration of 5 to 10 mg/ml of membrane protein. Erythrocyte plasma membranes (Bennett & Cuatrecasas, 1975*a*) from rats and toads, and choleragen-treated rat liver membranes (Bennett *et al.*, 1975) were prepared as described elsewhere.

Binding assays with ^{125}I-labeled cholera toxin were performed using filtration across cellulose acetate Millipore filters as described previously (Cuatrecasas, 1973*a*); the binding of the ^{125}I-labeled toxin to membranes was decreased to a value equal to that found in their absence by the prior addition of native toxin (10 µg/ml). The binding of ^{125}I-labeled glucagon to liver and fat cell membranes was determined in the presence of Bacitracin (1 mg/ml) (Desbuquois *et al.*, 1974) and assayed using the oil flotation method (Gliemann *et al.*, 1972). The binding of ^{125}I-labeled glucagon to membranes was reduced by 60 to 70% in the presence of native glucagon (1 µg/ml).

Adenylate cyclase activity was measured as described previously (Bennett & Cuatrecasas, 1975*a*), using chromatography on neutral alumina to isolate cyclic AMP (Ramachandran, 1971; White & Zenser, 1971). Unless otherwise stated, the assays were conducted in glass tubes (12 × 75 mm) in 0.1 ml containing 50 mM Tris-HCl, pH 8.0, 5 mM aminophylline, 5 mM phosphoenolpyruvate, 50 µg/ml pyruvate kinase (added as an ammonium sulfate suspension), 50 to 200 µg of membrane protein, 6.2 mM $MgCl_2$, 0.25 mM ATP, 0.2 mM GTP, and 4 to 8×10^6 cpm of [α-^{32}P]-ATP (10–20 Ci/mmole). The assays were terminated after 12 min at 30 °C by boiling the samples for 1 min. Assay blanks (boiled membranes) were usually less than 50 cpm per 10^6 cpm of total radioactivity added; basal activities were at least 500 cpm above the blank values. The activities varied linearly with membrane protein within the ranges employed. The time courses occasionally exhibited an accelerating phase between 0–4 min, but were linear at later times. Protein was estimated by the method of Lowry *et al.* (1951) after heating the samples for 40 min at 100 °C in 4 N NaOH; bovine serum albumin was used as the standard.

Results

Effect of Choleragen and (−)-Isoproterenol on the Response of Adenylate Cyclase of Rat Fat Cell Membranes to GTP and Magnesium

Incubation of isolated fat cells with low concentrations of choleragen (10^{-10} to 10^{-9} M) results in stimulation of adenylate cyclase activity and in conversion of the enzyme to a GTP-responsive state (Fig. 1*A, B*). The extent of stimulation by choleragen increases with increasing concentrations of GTP in the presence of high (1*B*) and low (1*A*) con-

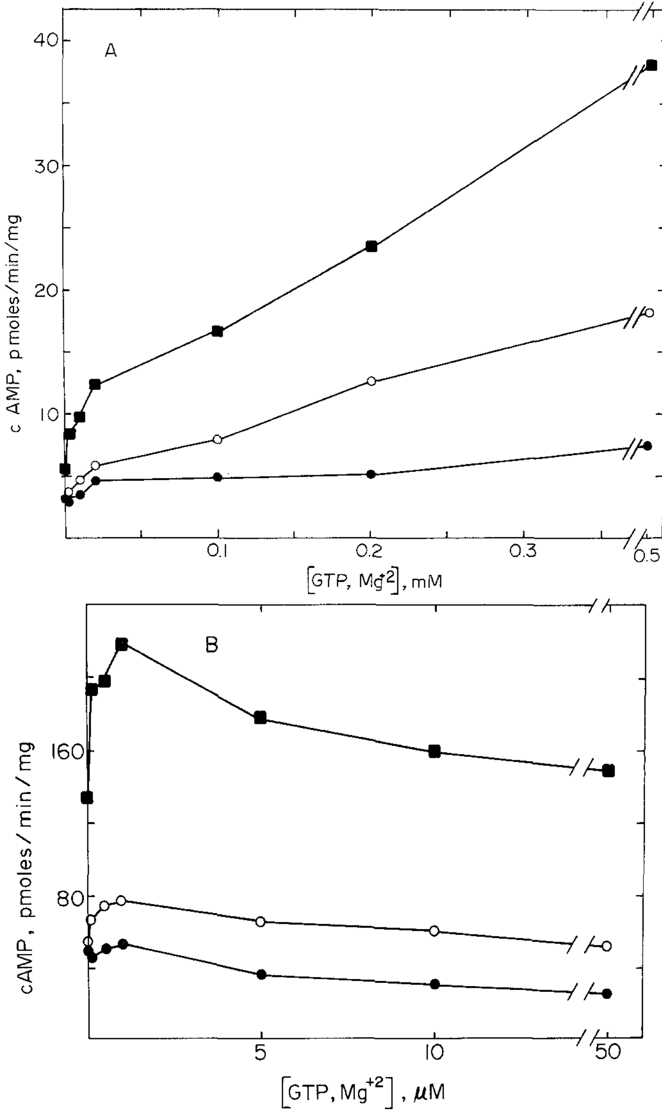

Fig. 1. Effect of increasing the concentration of 5′GTP, Mg^{2+} on the activity of adenylate cyclase of cholera toxin-treated (■) and untreated (●, ○) rat fat cell membranes assayed in the presence (○) and absence (●, ■) of (−)-isoproterenol. In panel *A*, $MgCl_2$ was present at stoichiometric equivalence to nucleotide triphosphates, while in panel *B*, $MgCl_2$ was present in 6 mM stoichiometric excess over the concentration of ATP and GTP. Isolated epididymal fat cells from eight 80 to 120 g rats (*see* Materials and Methods) were incubated at 37 °C in 10 ml of oxygenated Krebs-Ringer's bicarbonate, 2% (w/v) bovine serum albumin, pH 7.4, either in the absence (●, ○) or presence (■) of cholera toxin (0.1 μg/ml). After 75 min the cells were homogenized in Tris-HCl (50 mM, pH 7.7), and the 40,000 × *g* membrane pellets were assayed immediately for adenylate cyclase activity (12 min at 30 °C) in a 0.1 ml volume containing [α-^{32}P]-ATP (0.25 mM, 200 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), 50 to 60 μg of membrane protein, and increasing concentrations of GTP, $MgCl_2$. Some of the samples (○) also contained 10^{-7} M (−)-isoproterenol. $MgCl_2$ was present at 0.25 mM in *A*, and at 6.25 mM in *B*

centrations of Mg^{++}. A very similar pattern is observed when the enzyme activity is measured in the presence of (−)-isoproterenol (Fig. 1*A*, *B*) (Siegel & Cuatrecasas, 1974). In contrast, GTP has only a small effect on the basal enzyme activity (Fig. 1*A*,*B*). Since nucleoside triphosphates are known to complex magnesium (Izatt *et al.*, 1971), GTP was added with an equal amount of the metal. This practice, which has been adopted previously (Flawia & Torres, 1972; Siegel & Cuatrecasas, 1974), prevents the fall of free Mg^{++} with increasing amounts of nucleotide.

The GTP response of the toxin- and hormone-stimulated enzymes depends in a complicated way on the concentration of magnesium. In the presence of 6 mM Mg^{++}, half-maximal stimulation of the toxin- and hormone-treated enzymes occurs at about 10^{-7} M GTP (Fig. 1*B*). The activation by GTP is maximal at about 10^{-6} M, and higher concentrations of the nucleotide inhibit adenylate cyclase activity. A biphasic response to GTP in the presence of epinephrine has been observed previously in fat cell membranes (Siegel & Cuatrecasas, 1974). It is notable that the stimulation by toxin and (−)-isoproterenol *relative* to the control continues to increase even at GTP concentrations which inhibit the absolute activity values. Similar findings have been described for stimulation of fat cell adenylate cyclase by epinephrine, ACTH, and fluoride ion (Harwood & Rodbell, 1973). When Mg^{++} is present in stoichiometric equivalence to ATP and GTP (Fig. 1*A*), the apparent K_a for GTP is increased to a value greater than 0.2 mM for the toxin- and isoproterenol-stimulated enzymes. Also, in the presence of low metal, GTP stimulates at all concentrations.

The influence of magnesium on the GTP response of the catecholamine-stimulated adenylate cyclase activity has been reported before (Siegel & Cuatrecasas, 1974). It is possible that, in the presence of low magnesium, increasing amounts of GTP, $MgCl_2$ could result in an increase in the concentration of free Mg^{++} sufficiently high to activate the enzyme. In any event, the important findings from the standpoint of this study are that both the toxin- and hormone-activated enzymes are stimulated by GTP to a greater extent than untreated membranes and that they both demonstrate nearly identical response patterns to the nucleotide.

Effect of Choleragen on the Activation of Rat Fat Cell Adenylate Cyclase by (−)-Isoproterenol

Incubation of fat cells with choleragen for 70 to 90 min at 37 °C increases by about twofold the apparent affinity of adenylate cyclase for

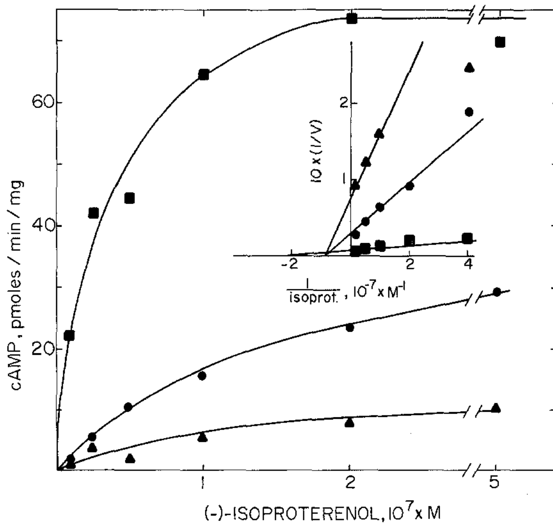

Fig. 2. Effect of incubating intact rat fat cells with cholera toxin (■) or (−)-isoproterenol (▲) on the apparent affinity of (−)-isoproterenol for activation of membrane adenylate cyclase. Fat cells from eight 80 to 120 g rats were incubated at 37 °C in 10 ml of oxygenated Krebs-Ringer's bicarbonate, 2% (w/v) bovine serum albumin, pH 7.4, either with no additions (●) or in the presence of 10^{-6} M (−)-isoproterenol (▲) or 0.1 μg/ml of cholera toxin (■). After 75 min the cells were homogenized in Tris-HCl (50 mM, pH 7.7) and the 40,000 × *g* membrane pellets were assayed for adenylate cyclase activity (12 min, 30 °C) (*see* Materials and Methods) in the presence of increasing concentrations of (−)-isoproterenol. The activity is expressed as hormone-stimulated activity (stimulated activity minus basal activity). The basal activities are 24 pmoles/min/mg (●), 241 pmoles/min/mg (■), and 36 pmoles/min/mg (▲)

activation by (−)-isoproterenol (Fig. 2). Although the effect is relatively small, it has been reproduced readily in at least eight experiments.

The change in the apparent affinity for (−)-isoproterenol is not explained by the fact that there is *in vivo* activation of adenylate cyclase during the incubation period since similar incubation of cells with (−)-isoproterenol does not significantly alter the subsequently measured affinity for the hormone (Fig. 2). Also, this increased sensitivity is not explained by the fact that the enzyme is activated during the assay since partial stimulation of the enzyme by glucagon does not lead to a change in the affinity of isoproterenol for further stimulation (not shown).

Choleragenoid, a biologically inert, competitive antagonist of choleragen binding and action (Cuatrecasas, 1973*d*; Pierce, 1973) does not influence the (−)-isoproterenol response of adenylate cyclase when it is incubated with fat cells under conditions identical to those used with choleragen (Fig. 3). Also, addition of choleragen to isolated fat cell membranes under conditions favorable for binding of native or ^{125}I-labeled

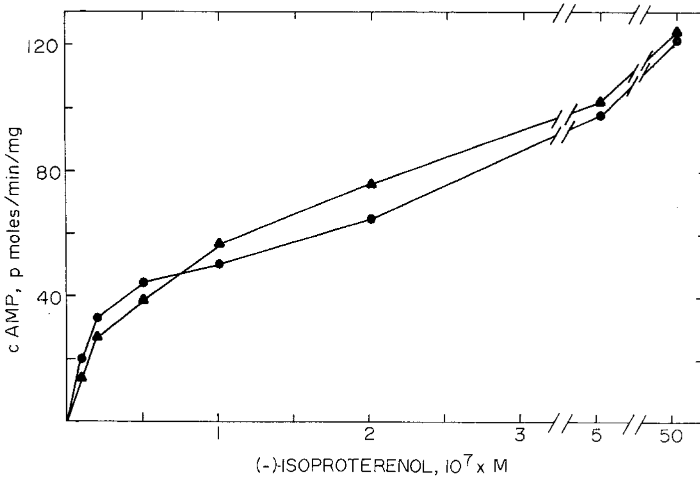

Fig. 3. Effect of incubating fat cells with choleragenoid (▲) on the apparent affinity of (−)-isoproterenol for activation of membrane adenylate cyclase activity. Isolated fat cells from six 80 to 100 g rats were incubated at 37 °C in oxygenated Krebs-Ringer's bicarbonate, 2% (w/v) BSA, pH 7.4, in the presence (▲) and absence (●) of 0.1 µg/ml choleragenoid. After 75 min the cells were homogenized in Tris-HCl (50 mM, pH 7.7) and the 40,000 × *g* membrane pellets were assayed for adenylate cyclase activity (12 min, 30 °C) in the presence of increasing concentrations of (−)-isoproterenol as described in Fig. 2. The data are presented as (−)-isoproterenol-stimulated activity (stimulated activity minus basal activity). The basal activites are 24 pmoles/min/mg (●) and 28 pmoles/min/mg (▲). Double-reciprocal plots of the data (not shown) indicate no significant difference in K_a for isoproterenol between control and toxin-treated membranes

toxin to membranes (Cuatrecasas, 1973*a*) does not affect enzyme activity or its response to (−)-isoproterenol (not shown). The altered hormone affinity is thus not caused solely by occupation of the binding sites for choleragen.

The increased affinity for isoproterenol is still observed when the cells are incubated with toxin in the presence of high concentrations of both actinomycin D and puromycin (Fig. 4). Thus, the effects most likely occur independently of possible changes in RNA and protein biosynthesis.

Incubation of cells with toxin may also increase the extent of adenylate cyclase activation by isoproterenol (Fig. 2). This effect of choleragen disappears when the cells are incubated for more than 90 min (37 °C). More prolonged exposure of the cells to choleragen results in such high stimulation (up to 30-fold) of basal enzyme activity that further activation by isoproterenol or other hormones is difficult to detect (not shown). The increase in apparent affinity for isoproterenol is still observed after extended periods of incubation of the cells with choleragen, but precise measurements of hormone stimulation are then quite difficult.

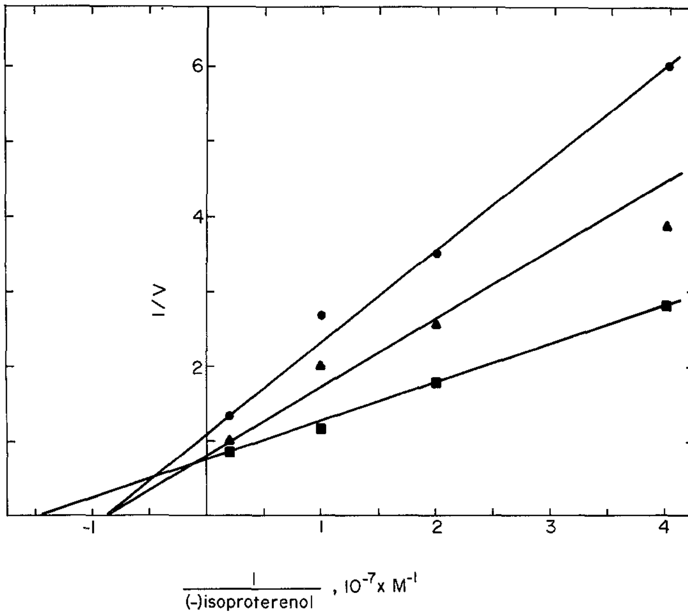

Fig. 4. Effect of inhibitors of protein and RNA synthesis on the choleragen-induced increase in the apparent affinity of (−)-isoproterenol for activation of adenylate cyclase activity of rat fat cell membranes. Fat cells were incubated at 37 °C in oxygenated Krebs-Ringer's bicarbonate, 2% albumin (w/v), pH 7.4, either with no additions (●) or in the presence of Actinomycin D (5 μg/ml) and puromycin (50 μg/ml) (▲), or in the presence of choleragen (0.2 μg/ml), Actinomycin D (5 μg/ml) and puromycin (5 μg/ml) (■). After 75 min, the cells were homogenized in Tris-HCl (50 mM, pH 7.7) and the 40,000 × *g* membrane pellets were assayed for adenylate cyclase activity (12 min, 30 °C) in the presence of various concentrations of (−)-isoproterenol as described in Fig. 2. The data are presented as double reciprocal plots of hormone-stimulated activity (stimulated activity minus basal activity) *vs.* hormone concentration. The basal activities are: 49 pmoles/min/mg (●); 61 pmoles/min/mg (▲); 144 pmoles/min/mg (■)

Effect of Choleragen on Activation of Adenylate Cyclase of Rat Fat Cells and Guinea Pig Ileal Mucosa by Polypeptide Hormones

Incubation of rat fat cells with choleragen for periods of 75 to 90 min (37 °C) causes a two- to fivefold increase in the apparent affinity for enzyme stimulation by ACTH, glucagon, and vasoactive intestinal peptide (Fig. 5 *A–C*). The magnitude of the increase in affinity for these hormones is similar to that observed with (−)-isoproterenol (Fig. 2). The maximal extent of glucagon stimulation of the toxin-treated enzyme appears to be increased relative to the response of the control enzyme; this effect, however, was not observed consistently with glucagon and was not found with ACTH or vasoactive intestinal polypeptide (5 *A, C*).

Choleragen also increases the apparent affinity of vasoactive intestinal polypeptide for activation of adenylate cyclase activity in guinea pig ileal mucosa membranes (Fig. 6). In this tissue the maximal extent of hormonal activation also appears to be increased by choleragen. The change in affinity for vasoactive intestinal polypeptide is of the same magnitude (about twofold) as observed in fat cells. These experiments involved injection of the toxin into ligated ileal loops of live animals, a situation which closely approximates that which occurs during enteric infection with *Vibrio cholerae* (De & Chatterje, 1953). The effects observed may have some relevance to the disease process.

Possible Interaction of Choleragen with Membrane Binding Sites for Hormones

The hormone-like effects of choleragen on the response of adenylate cyclase to GTP (Fig. 1), and the influence of toxin on the apparent affinity for hormones (Figs. 2, 5 and 6) might, in principle, result from direct interactions of the toxin with the binding sites for the hormones. Measurements of ^{125}I-labeled toxin binding, performed under conditions identical to those employed in the assay of adenylate cyclase activity, reveal that neither (−)-isoproterenol nor glucagon significantly affect the number or affinity of the binding sites for the toxin (Fig. 7). Furthermore, very high concentrations (5 μg/ml) of choleragen do not change the number or affinity of ^{125}I-labeled glucagon binding sites of liver and fat cell membranes (not shown) although membranes from toxin-treated cells bind glucagon with greater affinity than control membranes (Fig. 8; to be described). As described earlier, incubation of cells with the competitive antagonist of choleragen binding, choleragenoid, or incubation of membranes with choleragen itself, do not alter adenylate cyclase activity or its response to hormones.

The effects on choleragen-stimulated adenylate cyclase of propranolol and phentolamine, which are competitive antagonists of catecholamines and apparently bind in an inactive fashion to the receptor sites for these hormones (Levitzki *et al.*, 1974), were examined in amphibian erythrocytes since the enzyme of these cells responds only to catecholamines (Rosen & Rosen, 1969). High concentrations (10^{-4} M) of propranolol and phentolamine, either alone or in combination, do not significantly inhibit the basal adenylate cyclase activity of control or cholera toxin-stimulated membranes (Table 1). Propranolol, however, almost completely abolishes the epinephrine response of these membranes (Table 1).

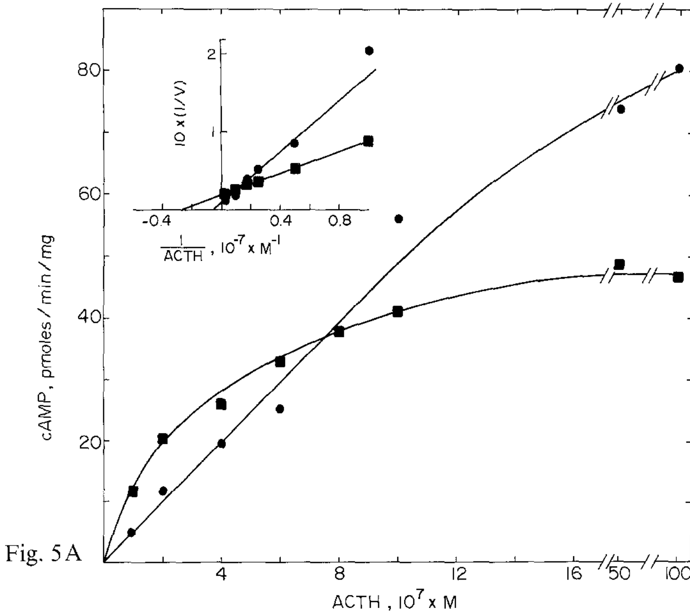

Fig. 5A

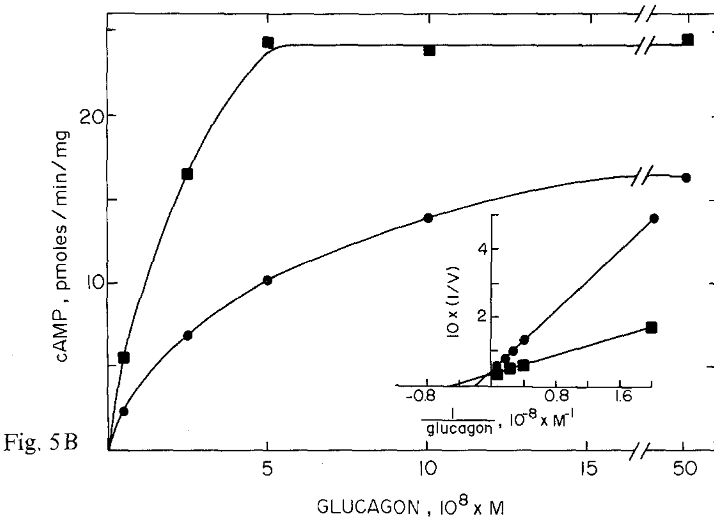

Fig. 5B

Fig. 5. Effect of incubating rat fat cells with choleragen on the apparent affinity of ACTH (*A*), glucagon (*B*) and vasoactive intestinal polypeptide (*C*) for activation of adenylate cyclase activity of membranes. Isolated fat cells from 80 to 140 g rats were incubated at 37 °C in oxygenated Krebs-Ringer's bicarbonate buffer as described in Figs. 2–4 in the presence (■) and absence (●) of choleragen (0.1 µg/ml). After 75 min, the cells were homogenized in Tris-HCl (50 mM, pH 7.7) and the 40,000 × *g* membrane pellets were assayed for adenylate cyclase activity (12 min at 30 °C) in the presence of various concentrations of hormones as described in Fig. 2. The activity is expressed as hormone-stimulated activity (stimulated activity minus basal activity). The basal activities are: Panel *A*-control, 28 pmoles/min/mg; toxin, 178 pmoles/min/mg. Panel *B*-control, 14.4 pmoles/min/mg, toxin, 84.9 pmoles/min/mg; Panel *C*-control, 7.6 pmoles/min/mg; toxin, 22.5 pmoles/min/mg

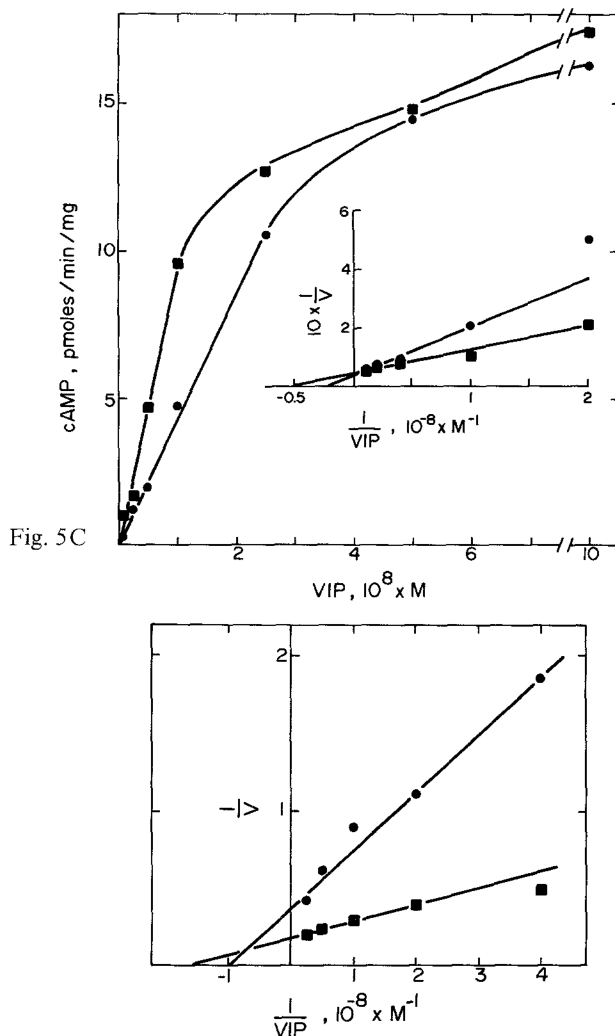

Fig. 6. Effect of incubating guinea pig ileum with cholera toxin on the apparent affinity of vasoactive intestinal polypeptide for activation of mucosal adenylate cyclase activity. A 10-cm section of ileum was ligated at both ends in each of two male guinea pigs (300 to 350 g) anesthetized with ether. The ileal loop of one animal was injected with 1 ml of Krebs-Ringer's bicarbonate, 0.1% (w/v) bovine serum albumin, pH 7.4 (●), while in the other animal it was injected with 1 ml of the same buffer containing choleragen (10 μg/ml) (■). The abdominal cavities were then closed and after 90 min the animals were sacrificed and the loops were excised and washed with Krebs-Ringer's bicarbonate, pH 7.4. The mucosa was collected by scraping, and membranes were prepared by homogenization (Brinkman polytron, 45 sec at a setting of 3.0) at 0 °C in 50 mM Tris-HCl, pH 7.7, 0.2 mM $CaCl_2$ and 5 μg/ml staphylococcal nuclease. The suspension was diluted fivefold with 50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.7, and the whole membrane fraction was isolated by centrifugation at 4 °C for 40 min at 40,000 × *g*. Adenylate cyclase activity (12 min at 30 °C) in the presence of increasing concentration of hormone was determined as described in Materials and Methods except that the concentrations ATP and GTP were 0.12 and 0.1 mM, respectively. The values were determined in triplicate and are expressed as double reciprocal plots of hormone-stimulated activity (stimulated activity minus basal activity) *vs.* the concentration of hormone. The basal activites are: control (●) 8 pmoles/min/mg; toxin-treated (■) 23 pmoles/min/mg

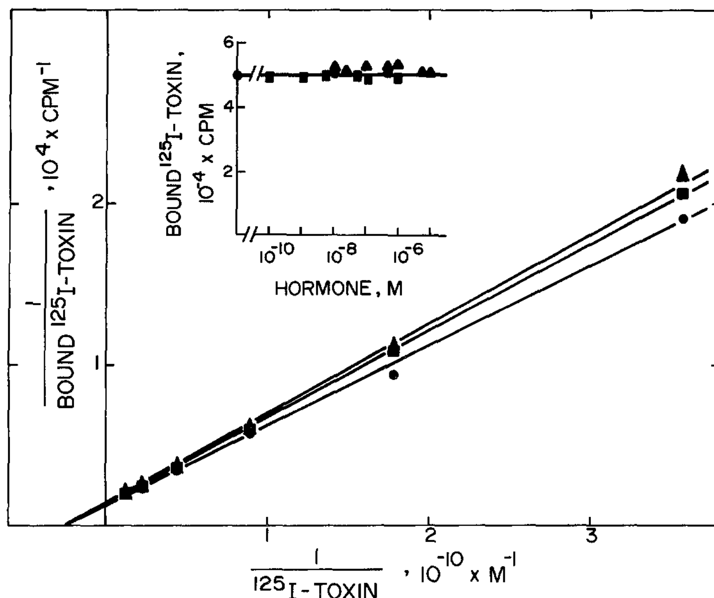

Fig. 7. Influence of glucagon (■) and (−)-isoproterenol (▲) on the specific binding of ^{125}I-labeled choleragen to fat cell membranes. Fat cell membranes were prepared and suspended in the medium (Figs. 2–6) utilized for the assay of adenylate cyclase (*see* Materials and Methods) except that [α-^{32}P]ATP was omitted. The specific binding of increasing concentations of ^{125}I-labeled choleragen (14 μCi/μg) was determined (*see* Materials and Methods) after incubating for 12 min at 30 °C with either no additions (●), or in the presence of 10^{-5} M glucagon (■) or 10^{-5} M (−)-isoproterenol (▲). The effect of various concentrations of glucagon and (−)-isoproterenol on the binding of a fixed concentration (2×10^{-10} M) of ^{125}I-labeled choleragen was also determined (*see* insert) under the same conditions. The values are expressed as cpm of ^{125}I-labeled choleragen bound specifically

Table 1. Effect of propranolol and phentolamine on adenylate cyclase activity of control and cholera toxin-treated toad erythrocyte plasma membranes[a]

Membrane preparation	Adenylate cyclase activity[b]			
	No additions	Propranolol	Phentolamine	Propranolol + phentolamine
Control	10 ± 1	13 ± 1	12 ± 1	12 ± 0.3
Control + (−)-epinephrine	17 ± 1	13 ± 1	15 ± 1	12 ± 1
Choleragen	123 ± 3	146 ± 11	140 ± 20	137 ± 6
Choleragen + (−)-epinephrine	384 ± 13	137 ± 8	314 ± 17	138 ± 3

[a] Cells were incubated in the presence and absence of choleragen (0.5 μg/ml) for 3 hr at 30 °C. Plasma membrane adenylate cyclase activity (10 min, 30 °C) was measured (*see* Materials and Methods) in the presence of $MgCl_2$ (6 mM), GTP (0.2 mM), [α-^{32}P]-ATP (0.6 mM, 66 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), 100 to 150 μg of membrane protein, and (−)-epinephrine (5×10^{-4} M), (+, −)-phentolamine (5×10^{-4} M), as indicated.

[b] pmoles of cAMP/min/mg of protein, mean of triplicate determinations ± one standard deviation.

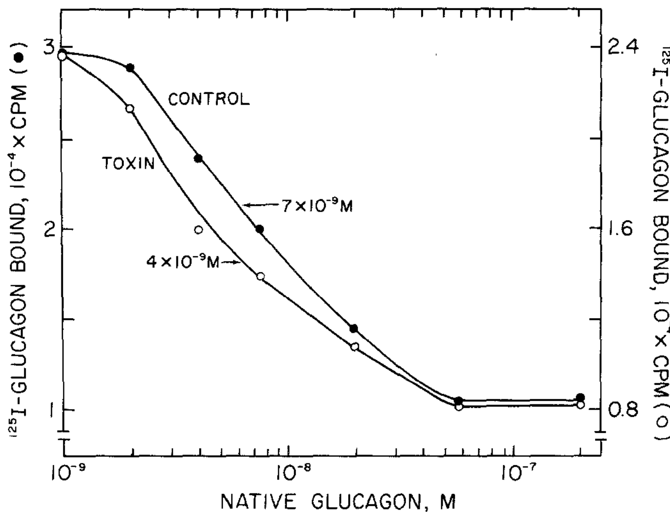

Fig. 8. Effect of cholera toxin treatment of liver on the apparent affinity of binding of ^{125}I-labeled glucagon to liver membranes. Sprague-Dawley rats were injected (intracardiac) with 100 μg of choleragen (○) or with Krebs-Ringer's bicarbonate (●) and 10 hr later the animals were sacrificed and heavy microsomal liver membranes were prepared (Chang *et al.*, 1975). Membranes (about 1 mg protein per ml) were incubated for 12 min at 4 °C in 0.2 ml of Krebs-Ringer's bicarbonate, pH 7.4, containing 0.1% (w/v) albumin, 0.1% (w/v) bacitracin, 250 μM GTP, 7×10^{-10} M (3.7×10^{5} cpm) ^{125}I-labeled glucagon and varying concentrations of native glucagon; the latter were added 10 min before addition of the ^{125}I-labeled hormone. Binding was determined by the oil flotation technique, as described elsewhere (Gliemann *et al.*, 1972; Bennett *et al.*, 1975)

Effect of Choleragen Treatment of Liver and Fat Cells on the Binding of ^{125}I-Labeled Glucagon

Although choleragen does not occupy hormone receptor binding sites, it is possible that the properties of the hormone-receptor interaction may be altered indirectly. The possible influence of choleragen treatment of liver and fat cells on the binding of ^{125}I-labeled glucagon to subsequently prepared membranes was therefore studied. Rat liver was treated with cholera toxin by intracardiac injection of the toxin (Gorman & Bitensky, 1972; Beckman *et al.*, 1974; Bennett *et al.*, 1975). The liver membranes isolated 10 hr later demonstrate a six- to 10-fold stimulation of adenylate cyclase activity relative to those of control animals, and the enzyme exhibits a twofold decrease in the rate of dissociation of membrane-bound glucagon (Bennett *et al.*, 1975).

Liver membranes from toxin-treated animals also show a twofold increase in the apparent affinity for binding of ^{125}I-labeled glucagon (Fig. 8). The total number of glucagon binding sites is not significantly altered

in these samples (not shown). This effect of choleragen treatment is probably not explained by an altered rate of glucagon degradation since the binding assays were conducted in the presence of bacitracin, an effective inhibitor of glucagon proteolysis (Desbuquois *et al.*, 1974). Toxin treatment of fat cells also increases the apparent affinity for ^{125}I-labeled glucagon binding (not shown). It has been reported previously that toxin treatment of liver decreases the rate of dissociation of the membrane-bound ^{125}I-labeled glucagon (Bennett *et al.*, 1975), which may explain the altered affinity for the hormone.

The effect of choleragen on the binding of ^{125}I-labeled glucagon to membranes requires prior incubation of intact cells with the toxin under conditions which elicit activation of adenylate cyclase. The apparent increase in the affinity of binding of ^{125}I-glucagon is relatively labile. Although the alterations persist following storage of membranes for 1 to 3 days at −190 °C, incubation at 20 °C for more than 30 min or at 0 °C for more than 4 hr, reverses these alterations in glucagon binding even though the toxin-stimulated state of adenylate cyclase is maintained.

Effect of Various Inhibitors and Diphtheria Toxin on the Activation of Adenylate Cyclase Activity by Choleragen

Choleragen activates adenylate cyclase and alters the affinity of the enzyme for hormonal stimulation only after incubation with intact cells for 20 to 40 min at 37 °C (not shown). It is not yet certain whether the delay or the activation process itself reflects intracellular events such as protein synthesis or processes related to microtubules or microfilaments.

Rat erythrocytes respond well to choleragen (Table 2) (Bennett & Cuatrecasas, 1974; 1975*a*) even though these cells lack a nucleus and have only a limited biosynthetic capacity. Moreover, erythrocytes are thought to be incapable of pinocytosis and phagocytosis (Hirsch *et al.*, 1973). Choleragen action in rat erythrocytes is not blocked by high concentrations of puromycin or Actinomycin D, either alone or in combination (Table 2)[2]. Also, the characteristic toxin-induced decrease in fluoride stimulation of cyclase activity (Bennett & Cuatrecasas, 1974; 1975*a, b;* Field, 1974) is still observed after treatment with these agents (Table 2).

To further explore the possible involvement of protein synthesis in choleragen action, studies were performed with diphtheria toxin, which

2 Such studies are very difficult to interpret in isolated fat cells because some of these compounds (especially puromycin) themselves have complicated effects when incubated with fat cells or when added directly to isolated membranes in the assay.

Table 2. Effect of drugs that disrupt microtubules and microfilaments and of inhibitors of protein and RNA biosynthesis on the ability of cholera toxin to activate adenylate cyclase in rat erythrocytes[a]

Incubation conditions		Adenylate cyclase activity[b]	
		Basal	NaF (10 mM)
Experiment I			
No additions:	control	1.8	35.5
	choleragen	10.4	23.1
Vincristine, 10^{-4} M:	control	1.1	17.9
	choleragen	15.4	21.5
Colchicine, 10^{-4} M:	control	1.7	26.4
	choleragen	13.1	25.8
Experiment II			
No additions:	control	2.2	33.2
	choleragen	5.4	13.5
Vinblastine, 10^{-4} M:	control	1.4	14.4
	choleragen	3.6	12.4
Cytocholasin B, 5 µg/ml:	control	1.6	19.4
	choleragen	3.5	10.1
Experiment III			
No additions:	control	1.2	13.7
	choleragen	6.1	13.7
Puromycin, 10 µg/ml:	control	1.4	14.1
	choleragen	6.4	12.8
Actinomycin D, 10 µg/ml:	control	0.9	9.6
	toxin	4.8	11.0
Puromycin, 10 µg/ml + actinomycin D, 10 µg/ml:	control	0.6	10.7
	toxin	5.1	10.4

[a] Washed cells (10 ml, about 10^8 cells per ml) were incubated in Krebs-Ringer's bicarbonate, pH 7.4, for 30 min at 37° C in the presence of various agents at the concentrations indicated. Choleragen (11 µg) was added to some of the samples, and the incubation was continued for three more hours at 37° C. The plasma membrane adenylate cyclase activity (18 min at 30 °C) was determined in the presence and absence of NaF as described in Materials and Methods except that the concentration of ATP was 0.12 mM.

[b] pMoles of cAMP formed per min per mg protein; mean value of triplicate determinations.

in most cells can block protein synthesis nearly completely (Strauss & Hendee, 1959). Cultured human fibroblasts (newborn foreskin) respond very well to choleragen even after exposure for 16 hr to concentrations of diphtheria toxin which abolish more than 98% of the incorporation of [^{14}C]-leucine into protein (Table 3) (Uchida *et al.*, 1973). Interestingly,

Table 3. Effects of diphtheria toxin on the stimulation of adenylate cyclase activity by cholera toxin in cultured human fibroblasts[a]

Cell treatment	Adenylate cyclase activity[b]	
	Basal	NaF (10 mM)
No additions	20	104
Cholera toxin (45 ng/ml for 3 hr)	351	273
Diphtheria toxin (17 ng/ml for 20 hr)[c]	86	151
Diphtheria toxin (17 ng/ml for 17 hr) followed by cholera toxin (45 ng/ml for 3 hr)	360	248

[a] Cultured fibroblasts (human newborn foreskin explant) were grown to confluency (about 2×10^7 cells per flask) in Eagles minimum essential medium, 10% (v/v) fetal calf serum, pH 7.4 (Hollenberg & Cuatrecasas, 1973). Two flasks of cells were incubated with diphtheria toxin (17 ng/ml) for 16 hr at 37 °C, as indicated. Some of the samples were then incubated with choleragen (45 ng/ml) for another 3 hr at 37 °C, and all of the flasks were harvested by scraping. Membranes were prepared by homogenization of the cell suspension with a Beckman Polytron (30 sec at a setting of 3.0) in a buffer (0 °C) containing Tris-HCl (5 mM, pH 8), $CaCl_2$ (0.2 mM), $MgCl_2$ (0.2 mM) and staphylococcal nuclease (1.5 μg/ml), followed by centrifugation (40 min at $40{,}000 \times g$). Adenylate cyclase activity was determined (12 min at 30 °C) in the presence and absence of NaF as described in Materials and Methods. In separate studies it was determined that under the conditions used here more than 98% of the [^{14}C] leucine incorporation into protein is inhibited by diphtheria toxin.

[b] Expressed as pmoles cAMP formed/min/mg protein; mean of duplicate determinations.

[c] Slight stimulatory effects of diphtheria toxin itself have been observed consistently.

diphtheria toxin itself activates adenylate cyclase activity, but to a much smaller extent than cholera toxin (Table 3).

The possible involvement of microtubules and microfilaments was tested in rat erythrocytes by using inhibitors such as colchicine, colcemide, vincristine, vinblastine, and cytocholasin B (Table 2). Even at high concentrations none of these compounds block the toxin-induced stimulation of adenylate cyclase activity or the inhibition of fluoride stimulation (Table 2).[3] It is also pertinent that these compounds and the inhibitors of protein synthesis do not themselves activate adenylate cyclase (Table 2).

Discussion

The purified exotoxin from *Vibrio cholerae* (choleragen) activates adenylate cyclase in a variety of tissues including the intestinal mucosa of many species (Sharp, 1973), rat fat cells (Evans *et al.*, 1972; Hewlett

3 These compounds also do not inhibit the lipolytic action of cholera toxin in fat cells (V. Bennett & P. Cuatrecasas, *manuscript in preparation*).

et al., 1974; Bennett & Cuatrecasas, 1975*b*), human neutrophils (Bourne *et al.*, 1973), mouse (Gorman & Bitensky, 1972) and rat liver (Beckman *et al.*, 1974; Bennett *et al.*, 1975), cultured mouse adrenal cells (Donta *et al.*, 1973; Wolff *et al.*, 1973); mouse melanocytes (O'Keefe & Cuatrecasas, 1974), thyroid tissue (Mashiter *et al.*, 1973), erythrocytes of rats (Bennett & Cuatrecasas, 1974, 1975*a*), turkeys (Field, 1974; Bennett & Cuatrecasas, 1975*b*) and toads (Bennett & Cuatrecasas, 1974; 1975*a, b*), and SV-40 transformed mouse fibroblasts (Hollenberg *et al.*, 1974).

The properties of the choleragen-stimulated adenylate cyclase of toad erythrocytes has been examined in detail recently (Bennett & Cuatrecasas, 1974; 1975*a*). Enzyme activation results from marked alterations in the regulatory characteristics of the enzyme. Choleragen and catecholamine hormones convert the enzyme to a purine nucleotide-sensitive state, such that low concentrations of ITP, GMP and GTP become stimulatory, whereas these nucleotides have little effect on the basal activity or in the presence of sodium fluoride (Bennett & Cuatrecasas, 1974, 1975*a*). Choleragen increases the response of toad erythrocyte adenylate cyclase to catecholamines, elevating the maximal extent of stimulation by three- to fivefold (Bennett & Cuatrecasas, 1974, 1975*a*). The toxin increases the maximal extent and apparent affinity for activation of turkey erythrocyte adenylate cyclase by catecholamines (Field, 1974). The increase in the apparent affinity for hormonal activation is also observed in toxin-treated mammalian cells, including catecholamine stimulation in rat erythrocytes (Bennett & Cuatrecasas, 1974, 1975*a*) and glucagon stimulation in rat liver membranes (Bennett *et al.*, 1975). Toxin-treated rat liver membranes also demonstrate a decreased rate of dissociation of membrane-bound ^{125}I-labeled glucagon (Bennett *et al.*, 1975).

The present studies demonstrate that in isolated rat fat cells choleragen increases the response of adenylate cyclase to low concentrations of GTP and that (−)-isoproterenol induces similar changes (Fig. 1). In the presence of low Mg^{++}, GTP activates the enzyme at all concentrations of the nucleotide (Fig. 1*A*). However, at high concentrations (6 mM) of the metal, GTP has a biphasic effect; the hormone and toxin-activated enzymes are stimulated in the range of 10^{-8} M to 5×10^{-6} M GTP (approximate K_d of 10^{-7} M), and inhibited at high concentrations. Similar findings have been reported previously for the (−)-epinephrine-stimulated cyclase of these cells (Siegel & Cuatrecasas, 1974).

Hormonally activated adenylate cyclase demonstrates unique responses to purine nucleotides. 5′GTP and 5′ITP preferentially activate the enzyme assayed in the presence of prostaglandins (Krishna *et al.*, 1972), glucagon

(Rodbell *et al.*, 1971), catecholamines (Leray *et al.*, 1972; Bilizekian & Aurbach, 1974; Siegel & Cuatrecasas, 1974), oxytocin (Bockaert *et al.*, 1972), and thyroid-stimulating hormone (Wolff & Cook, 1973). Adenylate cyclase assayed alone or in the presence of NaF is only slightly activated by GTP. The fact that a bacterial protein converts adenylate cyclase to a GTP-sensitive state is notable and suggests that some significant analogies may exist between the mechanism of adenylate cyclase activation by cholera toxin and hormones.

Cholera toxin increases the sensitivity of adenylate cyclase to hormonal stimulation. Treatment of fat cells with toxin induces a 1.5- to fivefold increase in the apparent affinity for adenylate cyclase activation by (−)-isoproterenol, ACTH, vasoactive intestinal polypeptide and glucagon. These effects are observed only under conditions of activation of adenylate cyclase by cholera toxin, i.e., only after an incubation period of at least 30 min at 37 °C.

Choleragen also increases the apparent affinity of vasoactive intestinal polypeptide for activation of adenylate cyclase in intestinal mucosa, the tissue which is affected in clinical cholera. This peptide hormone, which activates intestinal adenylate cyclase activity (Schwartz *et al.*, 1974), may have an important physiological role in the regulation of secretory functions in the small intestine (Barbeazat & Grossman, 1971). The increased sensitivity to this hormone could be of importance in the pathogenesis of cholera diarrhea. Physiological hormones which are themselves capable of generating diarrhea (Bloom *et al.*, 1973) may potentiate the effects of cholera toxin, especially since the luminal concentration of cholera toxin during infection may be quite small, as suggested by the low enterotoxicity of stool filtrates from cholera patients (Jenkin & Rowley, 1959).

The membrane binding properties of at least one hormone, glucagon, are also altered by cholera toxin. The apparent affinity of binding is increased (Fig. 8). The decreased rate of dissociation of membrane-bound ^{125}I-labeled glucagon in toxin-treated liver membranes (Bennett *et al.*, 1975) may perhaps explain the altered binding affinity reported here. Although it is tempting to relate the increased affinity of binding to the enhanced sensitivity (affinity) of adenylate cyclase stimulation by hormones, direct relationships remain to be established.

It is worthwhile to consider possible mechanisms by which choleragen alters the response of adenylate cyclase to purine nucleotides similarly to hormones and increases the affinity of adenylate cyclase for a variety of chemically distinct hormones. It seems unlikely that choleragen can interact directly with the binding sites for hormones. High concentrations

of glucagon and (−)-isoproterenol have no detectable effect on the binding ^{125}I-labeled cholera toxin (Fig. 7), and choleragen added directly to membranes does not affect hormonal stimulation of adenylate cyclase or glucagon binding even though the toxin binds avidly under these conditions. Furthermore, neither propranolol nor phentolamine (10^{-4} M) significantly affect toxin stimulation of adenylate cyclase in toad erythrocytes, cells which preseumably contain receptors only for catecholamines (Rosen & Rosen, 1969) (Table 1). It is conceivable that the initial toxin-membrane complex may involve a membrane structure which is a ubiquitous component of hormone receptors. This seems unlikely, however, since choleragenoid, a choleragen analog which binds to the identical membrane sites (Cuatrecasas, 1973*c*) and is a potent competitive antagonist of the biological effects of the toxin (Cuatrecasas, 1973*a*; Pierce, 1973) is completely inactive (Fig. 3) (Cuatrecasas, 1973*c*; Pierce, 1973; Bennett & Cuatrecasas, 1975*a*) and does not modify the effects of hormones (Fig. 3). Furthermore, an absolute delay of 15 to 30 min must occur before choleragen exerts any effects on the adenylate cyclase activity of intact cells. Simple occupation of the receptor sites for choleragen is thus not sufficient to affect adenylate cyclase activity.

The possibility that cholera toxin could function by altering some intracellular process has been considered unlikely. Direct measurements indicate negligible intracellular levels of ^{125}I-labeled choleragen after a 2- to 3-hr incubation period with toad erythrocytes (Bennett & Cuatrecasas, 1975*b*). Furthermore, the effects on adenylate cyclase activity and on the sensitivity of the enzyme to hormones are observed in the presence of metabolic inhibitors such as NaN_3, cycloheximide, actinomycin D, and puromycin (Bennett & Cuatrecasas, 1975*b*). Inhibitors of protein and RNA biosynthesis, and of prostaglandin synthesis and action, have no effect on the lipolytic response of rat fat cells to cholera toxin (Cuatrecasas, 1973*d*). Choleragen is fully active even when protein synthesis is completely abolished by diphtheria toxin (Table 3). Furthermore, the cholera toxin effects in enucleated rat erythrocytes (Bennett & Cuatrecasas, 1974; 1975*b*) and in rat fat cells are unaffected by inhibitors of protein and RNA synthesis (Fig. 4) or by agents which disrupt microtubules and microfilaments (Table 2).

Since hormonal modulation presumably involves, at some point, an association of hormone receptors with adenylate cyclase, it is also possible that direct interactions between the cyclase and choleragen molecules could occur and thus alter the properties of the hormone receptor-cyclase complex. For instance, the affinity of hormones could be greater for

receptors associated with and stabilized by the toxin-cyclase complex. It has been proposed (Cuatrecasas, 1974, 1975; Bennett *et al.*, 1975) that hormone receptors may exist separately from adenylate cyclase and that association between these molecules requires lateral diffusion within the two-dimensional matrix of the plasma membrane. If this were the case, the toxin-cyclase complex could perhaps interact with hormone receptors more efficiently, and the specific properties of those receptors could, for the same reasons, be altered.

Recent direct evidence has been presented which shows that incubation of fat cells with choleragen results in the formation of a detergent-stable complex between adenylate cyclase and some portion of the toxin molecule (Bennett *et al.*, 1975). Furthermore, the kinetics of cyclase activation, and the concentration-response relationships strongly suggest that a bimolecular association occurs between membrane-bound cholera toxin and another membrane component, presumably adenylate cyclase (Bennett & Cuatrecasas, 1975*b*). It has been proposed (Bennett & Cuatrecasas, 1975*a, b;* Bennett *et al.*, 1975) that the "active" (hydrophobic) subunit (Cuatrecasas *et al.*, 1973; Lonnroth & Holmgren, 1973; van Heyningen, 1974) of choleragen may become incorporated into the lipid phase of the plasma membrane, becoming an "integral" (Singer & Nicolson, 1972) membrane protein, where it may complex with and stimulate adenylate cyclase in the absence of hormone. This mechanism could explain how cholera toxin can universally stimulate and modify an enzyme which is normally regulated by hormones with a high degree of specificity.

This research was supported by grants from the National Institutes of Health (AM 14956), the National Science Foundation (GB-34300) and the Kroc Foundation. V.B. is a recipient of a Medical Scientist Scholarship funded by Home Life Insurance Co. P.C. is a recipient of a USPHS Research Career Development Award AM 31464.

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