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Mechanism of Action of *Vibrio cholerae* Enterotoxin

Effects on Adenylate Cyclase of Toad and Rat Erythrocyte Plasma Membranes*

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Summary. The characteristics of the cholera toxin-stimulated adenylate cyclase of toad (*Bufus marinus*) and rat erythrocyte plasma membranes have been examined, with special emphasis on the response to purine nucleotides, fluoride, magnesium and catecholamine hormones. Toad erythrocytes briefly exposed to low concentrations of cholera toxin (40,000 to 60,000 molecules per cell) and incubated 2 to 4 hr at 30 °C exhibit dramatic alterations in the kinetic and regulatory properties of adenylate cyclase. The approximate K_m for ATP, Mg^{++} increases from about 1.8 to 3.4 mM in the toxin-stimulated enzyme. The stimulation by cholera toxin increases with increasing ATP, Mg^{++} concentrations, from 20% at low levels (0.2 mM) to 500% at high concentrations (greater than 3 mM). Adddition of GTP, Mg^{++} (0.2 mM) restores normal kinetic properties to the toxin-modified enzyme, such that stimulation is most simply explained by an elevation of V_{max}. GTP enhances the toxin-treated enzyme activity two- to fourfold at low ATP concentrations, but this effect disappears at high levels of the substrate. At 0.6 mM ATP and 5 mM $MgCl_2$ the apparent K_a for GTP, Mg^{++} is 5 to 10 μM. The control (unstimulated) enzyme demonstrates a very small response to the guanyl nucleotide. 5′-ITP also stimulates the toxin-treated enzyme but cGMP, guanine, and the pyrimidine nucleotides have no effect. Cholera toxin also alters the activation of adenylate cyclase by free Mg^{++}, decreasing the apparent K_a from about 25 to 5 mM. (−)-Epinephrine sensitizes the toad erythrocyte adenylate cyclase to GTP and also decreases the apparent K_a for free metal. Sodium fluoride, which causes a 70- to 100-fold activation of enzyme activity, has little effect on sensitivity to GTP, and does not change the apparent K_a for Mg^{++}; moreover, it prevents modulation of these parameters by cholera toxin. Conversely, cholera toxin severely inhibits NaF activation, and in the presence of fluoride ion the usual three- to fivefold stimulation by toxin becomes a 30 to 60% inhibition of activity. The toxin-stimulated enzyme can be further activated by catecholamines; in the presence of GTP the (−)-epinephrine stimulation is enhanced by two- to threefold. The increased catecholamine stimulation of toad erythrocyte adenylate cyclase induced by cholera toxin is explained primarily by an increase in the maximal

* Part of this work was reported at the 1974 meeting of the Federation of American Societies for Experimental Biology (Bennett & Cuatrecasas, 1974).

extent of activation by the hormones. Rat erythrocyte adenylate cyclase is also modified by cholera toxin. In the mammalian system the apparent affinity for the hormone appears to be increased. Cholera toxin thus induces profound and nearly permanent changes in adenylate cyclase by a unique process which mimics the stimulation by hormones in important ways, and which also accentuates the normal hormonal response. The relevance of these findings to the mechanism of action of cholera toxin is considered.

Cholera toxin, an enterotoxin from *Vibro cholerae* which has recently been purified to homogeneity (Finkelstein & LoSpalluto, 1969, 1970), stimulates cyclic AMP-mediated metabolic processes in all tissues examined so far (reviewed by Finkelstein, 1973). These effects have been correlated with activation of adenylate cyclase in the intestinal mucosa of many species (reviewed by Sharp, 1973), mouse liver (Gorman & Bitensky, 1972), rat fat cells (Evans, Chen, Carlin & Evans, 1972), human neutrophils (Bourne *et al.*, 1973), cultured mouse adrenal tumor cells (Donta, King & Sloper, 1973; Wolff, Temple & Cook, 1973), mouse melanocytes (O'Keefe & Cuatrecasas, 1974), rat thymocytes (Boyle & Gardner, 1974), and thyroid tissue (Mashiter, Mashiter, Hauger & Field, 1973). Cholera toxin has no influence on cAMP phosphodiesterase activity under conditions where adenylate cyclase activity is clearly stimulated (Kimberg *et al.*, 1971).

Cholera toxin exhibits some unusual biological properties that make this protein unique among other known modulators of adenylate cyclase activity. Only a brief exposure to the toxin is required for the full expression of the biological effects, and these cannot be reversed by extensive washing of the cells (van Heyningen *et al.*, 1971; Cuatrecasas, 1973*c*) or by the addition of specific antibody (Mosely & Ahmed, 1969) or other known inhibitors of cholera toxin binding to cell membranes (Cuatrecasas, 1973*c*, *d*; Wolff *et al.*, 1973). Despite the rapidity of the initial toxin-cell interaction, a lag period of at least 30 min must occur before any effects are observed (Vaughan, Pierce & Greenough, 1970; Kimberg *et al.*, 1971; Cuatrecasas, 1973*c*). Cholera toxin is effective only if it is incubated with intact cells, although once adenylate cyclase activation has occurred, this is not reversed by cell lysis or plasma membrane isolation procedures. Studies with [^{125}I]-cholera toxin (Cuatrecasas, 1973*a*, *b*, *d*; Boyle & Gardner, 1974; Hollenberg, Fishman, Bennett & Cuatrecasas, 1974; Holmgren, Lindholm & Lonnroth, 1974; Walker, Field & Isselbacher, 1974) have demonstrated that the toxin binds rapidly and with high affinity to cell membranes, and that the binding site is most likely a specific glycolipid, G_{M1} ganglioside. Other workers have independently postulated a receptor role for this ganglioside (Holmgren, Lonnroth & Svennerholm, 1973; King & van Heyningen, 1973; van Heyningen, 1973, 1974).

The present report describes the properties of the cholera toxin-activated adenylate cyclase of toad and rat red blood cells. These erythrocytes were chosen because they provide a simple system of homogeneous, isolated cells with limited metabolic capacity, and they contain a hormone-sensitive adenylate cyclase (Rosen & Rosen, 1968, 1969; Sheppard & Burghardt, 1969, 1970). It is demonstrated that cholera toxin dramatically alters the regulatory properties of the amphibian adenylate cyclase in a way quite similar to the behavior of hormones in many other tissues, and of catecholamines in these cells. Furthermore, it is shown that cholera toxin modifies the response of adenylate cyclase to catecholamines primarily by increasing the maximal hormonal stimulation (toad cells) or by increasing the apparent affinity for these hormones (rat cells).

Experimental Procedure

Materials

Cholera toxin (lot # 1071) and cholera toxoid (lot # G 0673), purified by the method of Finkelstein and LoSpalluto (1970), were obtained from Dr. C. E. Miller, SEATO Cholera Research Program. Tetanus toxin was obtained from Burroughs-Wellcome. 5′ATP, 5′GTP, cGMP, phosphoenolpyruvate, (−)-epinephrine bitartrate, (−)-isoproterenol HCl, propranolol HCl, phentolamine HCl, and neutral alumina were purchased from Sigma, 5′ITP, 5′XMP, 5′CTP, 5′UTP, 5′TTP, and 5′GDP from Miles, 5′GMP and guanosine from Calbiochem, 2′,3′isopropylidene adenosine and 2′,3′isopropylideneguanosine from Aldrich, pyruvate kinase and myokinase from Boehringer, staphylococcal nuclease (6000 U/mg) from Worthington, and [^{32}P]-orthophosphoric acid (carrier-free) in 0.02 N HCl and [^{3}H]-cAMP (22 Ci/mmole) were from New England Nuclear. Carrier-free Na^{125}I, in 0.1 N NaOH was obtained from Union Carbide. [γ-^{32}P]ATP (40–50 Ci/mmole) was prepared by the method of Glynn and Chappel (Glynn & Chappel, 1964).

Preparation of [α-^{32}P]ATP

[α-^{32}P]ATP (20–60 Ci/mmole) was synthesized according to the method of Symons (Symons, 1968; Flawia & Torres, 1972*a*) and purified by descending chromatography on Whatman 3 MM paper with ethanol, ammonium acetate (1.0 M, pH 7.0, 2.5:1 v/v), NaEDTA (0.1 mM), as a solvent. After 15–18 hr at 4 °C, the paper was washed for 2–4 hr with ethanol, and dried. The strip containing labeled ATP was eluted with 25 μM NaEDTA, pH 8.3, and stored at a concentration of 3–5 mCi/ml in small aliquots at −20 °C. The radiopurity of the final product is usually greater than 98%, based on the chromatographic behavior on PEI cellulose thin-layer strips in 0.2 M NH_4HCO_3. Assay blanks with freshly prepared ATP are generally less than 0.005% of the total radioactivity.

Preparation of Toad Erythrocyte Plasma Membranes

Heparinized whole blood was obtained by cardiac puncture from one or two females (*Bufus marinus*) weighing 100–200 g. The serum and buffy coat were removed by repeated washes (6–10 times) with a buffer (amphibian Ringer's) containing $MgCl_2$ (1 mM), $CaCl_2$ (1.2 mM), KCl (1.9 mM), $NaHCO_3$ (2.4 mM), NaCl (120 mM), at a pH of 7.5. By

phase microscopy the erythrocytes contain less than 0.5% contamination by other cell types. Nucleated ghosts are formed by hypoosmotic shock in the presence of low concentrations (0.5 μg/ml) of highly purified staphylococcal nuclease; this enzyme is required to prevent irreversible clumping caused by the release of nucleic acids during cell lysis (Rosen & Rosen, 1969). Twenty μg of nuclease (20 μliters of a 1 mg/ml solution) are added to 1 ml of packed erythrocytes, and the cells are lysed by the rapid introduction (from a syringe) of 40 ml of ice-cold 5 mM Tris-HCl, 0.1 mM $CaCl_2$, pH 8.0. After 10–15 min at 0 °C, the ghosts are enucleated by shaking the tubes vigorously for about 10 sec. The free nuclei and remaining nucleated ghosts are pelleted by centrifugation at $2000 \times g$ for 10 min at 0 °C. The supernatant, containing the plasma membranes, is made 0.5 mM in $MgCl_2$ (to facilitate sedimentation) and centrifuged at $10^5 \times g$ for 20 min at 0 °C in a swinging bucket rotor (Beckman SWL-27). The final pellet is resuspended in 50 mM Tris-HCl, pH 8.0, to a concentration of 5–10 mg/ml membrane protein, and stored in ice until assay. Phase-contrast microscopy reveals open membrane sacs of about the same dimensions as the intact cells. Human and rat erythrocyte plasma membranes were prepared by essentially the same procedure, except that no nuclease is required. NaCl (20–50 mM) must be added to the cell lysate in order to obtain a firm membrane pellet.

Assay of Adenylate Cyclase Activity

This was determined in a 0.1 ml volume 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), 100–200 μg of membrane protein, various concentrations of $MgCl_2$, 5′GTP, and 5′ATP, and 4–8 million cpm of [α^{32}P]ATP (20–60 Ci/mmole). All additions were made at 0 °C, and the reaction was initiated by transfer of the samples to a 30 °C water bath. The assay was terminated by boiling the tubes for 1–2 min. 1.0 ml of a recovery mixture containing Tris-HCl (25 mM, pH 7.6) and [^{3}H]cAMP (20,000–40,000 cpm) was added to each sample, which was then applied to a column containing 1 g of dry, neutral alumina (Ramachandran, 1971; White & Zenser, 1971). cAMP was isolated by eluting the columns with 2.0 ml of Tris-HCl (25 mM, pH 7.6) directly into counting vials containing Bray's solution (12 ml) (Bray, 1960). The recovery of [^{3}H]-cAMP (determined for each sample) averaged 75–80%. Assay blanks (boiled membranes) were usually less than 50 cpm per 10^6 cpm of total radioactivity; basal activities were at least 500 cpm above the blank values. The product obtained after alumina columns co-migrates with [^{3}H]-cAMP in several TLC and ion exchange column chromatographic systems.

Procedures

Cholera toxin was labeled with ^{125}I as described previously (Cuatrecasas, 1973*a*) using a modification (Cuatrecasas, 1971) of the method of Hunter and Greenwood (1962). Before use, cholera toxin (5 mg in 0.2 ml) was passed through a G-75 Sephadex column equilibrated with 0.25 M sodium phosphate, pH 7.4. The binding of [^{125}I]-toxin (Cuatrecasas, 1973*a*) to toad erythrocytes is decreased to a value equal to that found in the absence of cells (less than 1% of the total radioactivity) by prior addition of native toxin (10 μg/ml) to the cells. Membrane protein was determined by the method of Lowry, Rosebrough, Farr and Randall (1951) after heating the samples for 40 min at 100 °C with 4 N NaOH. Bovine serum albumin was used as the standard.

($Na^+ + K^+ + Mg^{2+}$)-ATPase activity was determined in the presence of NaCl (120 mM), KCl (10 mM), $MgCl_2$ (2 mM), [γ-^{32}P]-ATP (2 mM, 3500 cpm/nmole), Tris-HCl (50 mM, pH 7.4) and 10–20 μg of membrane protein. The reaction was terminated by

boiling for 1 min, and the remaining ATP precipitated with Norite (Avruch & Wallach, 1971). Assay blanks (boiled membranes) were 0.1–0.2% of the total radioactivity. The hydrolysis of [γ-^{32}P]-ATP was linear up to 15 min at 23 °C.

Results

General Properties of the Cholera Toxin-Modified Adenylate Cyclase of Toad Erythrocyte Plasma Membranes

Cholera toxin, after preincubation with intact cells, clearly alters the adenylate cyclase activity of plasma membrane preparations (Table 1). The basal enzyme activity is elevated three- to fivefold, the response to catecholamines is doubled, and the relative NaF stimulation is inhibited. The increased catecholamine response magnifies the effect of the toxin from a three- to fivefold increase in the unstimulated enzyme to a 10- to 20-fold

Table 1. Adenylate cyclase activity of toad erythrocyte plasma membranes after preincubation of the cells with cholera toxin, choleragenoid, and (−)-epinephrine[a]

Cell treatment	Adenylate cyclase activity[b]			
	No additions	(−)-epinephrine	NaF	NaF and (−)-epinephrine
Control	8	17	621	605
Cholera toxin	46	231 150[c]	423	444
Choleragenoid	8	16	589	—
Cholera toxin + choleragenoid	15	62	531	—
(−)-Epinephrine	10	21	617	593

[a] Washed erythrocytes were suspended at 0 °C in amphibian Ringer's and divided into five 50-ml portions, each containing 1.8×10^8 cells. Two of these were exposed (20 min, 0 °C) to either cholera toxin (5.4 µg) or choleragenoid (5.6 µg). One was exposed (20 min, 0 °C) to choleragenoid (5.6 µg) before addition of cholera toxin (5.4 µg). All five groups of cells were washed twice, resuspended in 20 ml of Ringer's, and incubated at 30 °C. (−)-Epinephrine (2×10^{-4} M) was added to one tube 45 min before termination of the incubation period. After 4 hr, the cells were pelleted and the plasma membrane adenylate cyclase activity was measured (*see* Experimental Procedure) at 30 °C for 12 min in the presence of $MgCl_2$ (6 mM), GTP (0.2 mM), [α-^{32}P]-ATP (0.6 mM, 104 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 µg/ml), Tris-HCl (50 mM, pH 8.0), 70-100 µg of membrane protein, and, where indicated, sodium fluoride (10 mM) or (−)-epinephrine (10^{-4} M).

[b] pMoles of cAMP/min/mg protein, mean value of triplicate determinations; standard deviations were less than 6%.

[c] Medium containing (−)-epinephrine used in the preincubation of intact cells, diluted 1:4 in final assay mixture.

enhancement in the presence of the hormone. Toxin paradoxically inhibits adenylate cyclase activity when assayed in the presence of NaF.

The stimulation of basal enzyme activity by cholera toxin is not inhibited by propranolol or phentolamine (10^{-4} M), either alone or in combination *(unpublished observation)*. Propranolol, however, almost completely abolishes the (−)-epinephrine stimulation of both toxin-exposed and untreated membranes while phentolamine has only a small effect *(unpublished observation)*. It is unlikely that some of the alterations observed with cholera toxin are due to a change in ATPase activity. The assays were conducted with an efficient ATP regenerating system. Furthermore, direct measurements of the $(Na^+ + K^+ + Mg^{2+})$-ATPase activity indicated no difference between toxin-treated and control membranes. The effects of cholera toxin described above require the intact toxin molecule. Choleragenoid, a potent competitive antagonist (Cuatrecasas, 1973*d*; Pierce, 1973) which lacks a 36,000 MW subunit (Holmgren, Lonnroth, Ouchterlony & Svennerholm, 1972; Cuatrecasas, Parikh & Hollenberg, 1973; Lonnroth & Holmgren, 1973; Finkelstein *et al.*, 1974) has no effect when preincubated with erythrocytes (Table 1), even though this molecule binds with very high affinity to the identical membrane sites as cholera toxin (Cuatrecasas, 1973*d*). On the other hand, brief exposure to choleragenoid blocks 70% of the subsequent response to nearly equimolar concentrations of toxin (Table 1). High concentrations (1.7 μg/ml) of tetanus toxin, which binds to G_{D1} and G_{T1} gangliosides (van Heyningen & Miller, 1961; van Heyningen & Mellanby, 1968) are also inert in this system (data not shown).

The time course of the adenylate cyclase reaction is linear between 6 and 18 min, and no differences are seen in the time course pattern of the basal or epinephrine-stimulated activities in the normal or toxin-treated membranes (Fig. 1). The inhibitory effect of toxin on the fluoride-stimulated enzyme follows a very similar time course (Fig. 2). The apparent "lag" or accelerating phase of enzyme activation which is observed before linear (zero order) kinetics are achieved is seen consistently. Similar delays, described recently by Rodbell, Lin and Salamon (1974) in hormone activation of liver adenylate cyclase, were ascribed to the relatively slow process of hormone binding to receptors. It should be noted, however, that such "lag phases" are seen in the basal as well as NaF-stimulated activity in the presence or absence of cholera toxin.

The effects of cholera toxin do not result simply from prolonged *in vivo* activation of adenylate cyclase, since preincubation of intact cells with (−)-epinephrine under conditions identical to those used with cholera toxin does not elicit these changes (Table 1). This is not due to oxidation and loss of epinephrine from the medium since the incubation medium was capable

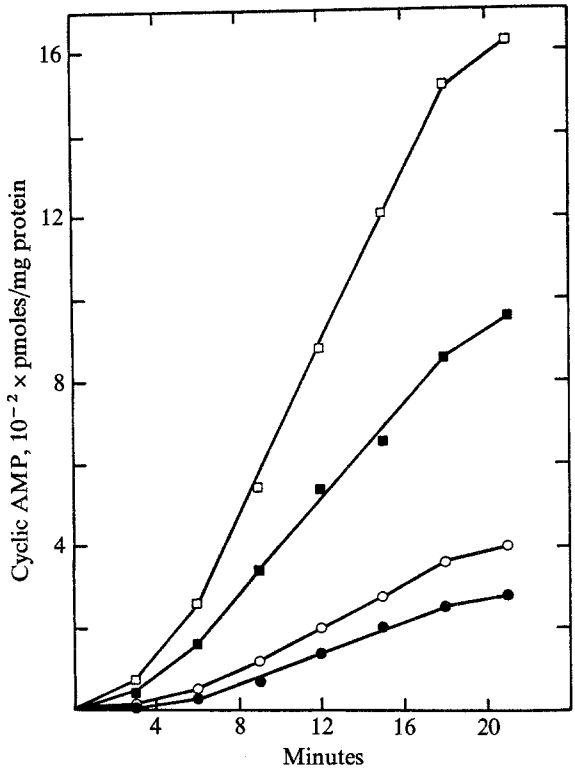

Fig. 1. Time course of cyclic AMP production by control (●, ○) and cholera toxin-treated (■, □) toad erythrocyte plasma membranes assayed in the presence (□, ○) and absence (■, ●) of (−)-epinephrine. Washed erythrocytes were divided into two portions, each containing about 2×10^9 cells, and suspended in 30 ml of amphibian Ringer's. Cholera toxin (10.8 μg) was added to one of these, and both groups of cells were incubated for 4 hr at 30 °C. The cells were then pelleted, and the plasma membrane adenylate cyclase assayed (*see* Experimental Procedure) in the presence of $MgCl_2$ (5 mM), [α-^{32}P]-ATP (1.2 mM, 34 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0) and 220–240 μg of membrane protein. Some tubes also contained (−)-epinephrine (10^{-6} M). The samples were incubated for various times at 30 °C. The cyclic AMP produced by both control and toxin-treated plasma membranes exhibits a linear increase with protein up to 250 μg when assayed for 15 min at 30 °C under the conditions described in this Figure

of stimulating adenylate cyclase when assayed 2 hr later (Table 1). It is unlikely that cholera toxin acts by "stabilizing" of adenylate cyclase since incubations for as long as 5 hr do not significantly alter the basal activity, catecholamine sensitivity, or sodium fluoride stimulation *(unpublished data)*. The persistent nature of the toxin effect is illustrated by the fact that erythrocytes exposed only briefly (4 °C, 20 min) to low concentrations of cholera toxin (a total of 40,000–60,000 molecules added per cell) exhibit profound changes in adenylate cyclase activity 4–6 hr later. Cell lysis and plasma

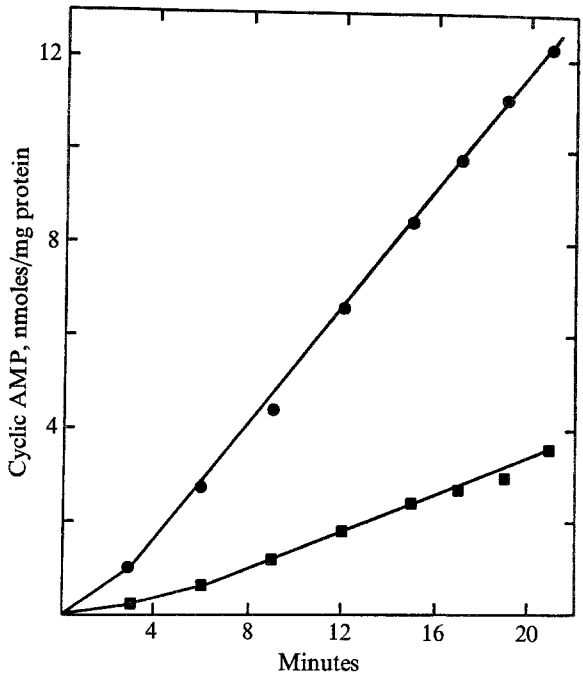

Fig. 2. Effect of sodium fluoride on adenylate cyclase activity of control (●) and cholera toxin-treated (■) toad erythrocyte plasma membranes. Erythrocytes were washed and incubated in the presence and absence of cholera toxin as described in Fig. 1. Adenylate cyclase activity was measured (*see* Experimental Procedure) in the presence of NaF (20 mM), $MgCl_2$ (2.5 mM), [α-^{32}P]-ATP (1.2 mM, 43 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0) and 190–200 μg of membrane protein. The samples were incubated for various times at 30 °C, and then boiled for 1 min. The values were determined in triplicate

membrane isolation apparently do not destroy the toxin effects. Moreover, the basic kinetic properties of the toxin-treated enzyme do not change with prolonged incubation of the isolated membranes (Figs. 1 and 2).

Cholera toxin must be incubated with intact erythrocytes for at least 30 min before any change in adenylate cyclase activity is observed *(unpublished data)*. Cell disruption not only prevents the action of subsequently added toxin but also interrupts the process of activation by cholera toxin previously bound to the intact erythrocytes. The kinetics and properties of the action of toxin on intact cells are discussed in detail in the accompanying paper (Bennett & Cuatrecasas, 1975).

Binding of [^{125}I]-Cholera Toxin to Toad Erythrocytes

^{125}I-Derivatives of cholera toxin (spec. act. 1–2 Ci/μmole) bind to intact toad erythrocytes (Fig. 3) in a manner nearly identical to that described for

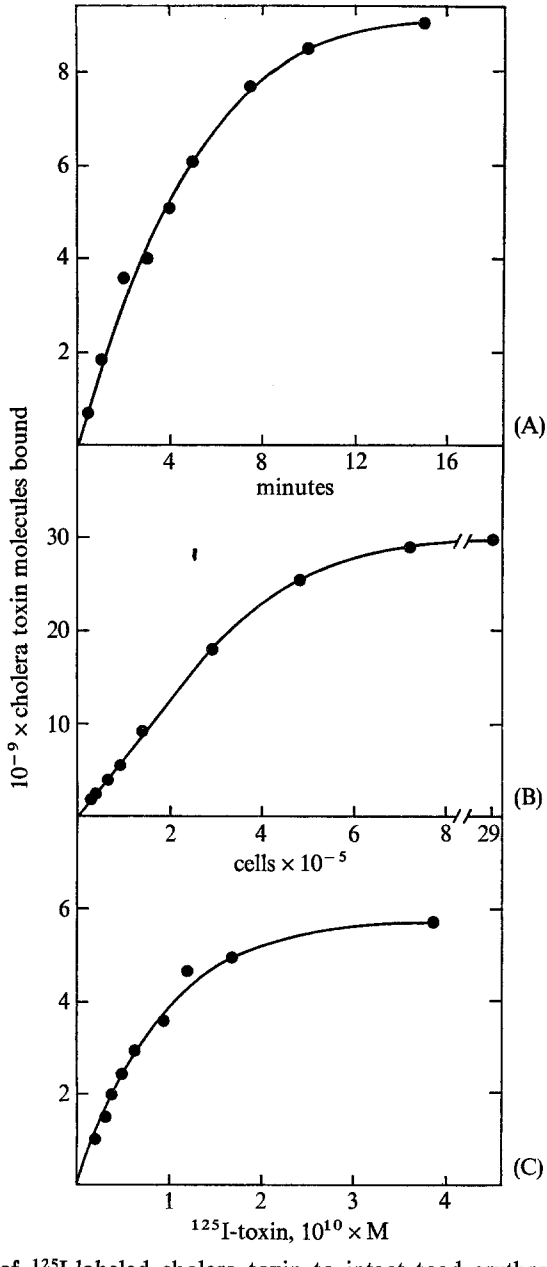

Fig. 3. Binding of ^{125}I-labeled cholera toxin to intact toad erythrocytes. Binding (*see* Experimental Procedure) was performed at 4 °C with thoroughly washed erythrocytes suspended in amphibian-Ringer's, 0.1 % (w/v) BSA, pH 7.5. The time course of binding of [^{125}I]-toxin (2.6×10^{-10} M, 1.3 Ci/μmole) was measured in the presence of 1.4×10^5 cells in a volume of 0.24 ml (panel *A*). The effect of cell number on the binding of[^{125}I]-toxin (2.6×10^{-10} M) was determined after incubating for 45 min (4 °C) in a volume of 0.24 ml (panel *B*). The effect of increasing the concentration of [^{125}I]-toxin was measured (40 min, 4 °C) using 7.2×10^4 cells in a volume of 0.5 ml (panel *C*). The molecular weight and $A^{1\%}_{1\,cm}$ (280 nm) of cholera toxin were assumed to be 90,000 and 11.4, respectively (LoSpalluto & Finkelstein, 1972). The total number of binding sites per erythrocyte was estimated from the slope of the linear portion of the curve in panel *B*. This value varied with different samples of blood but was in the range of 20,000–100,000 sites per cell

rat fat cells (Cuatrecasas, 1973*a*). The binding is rapid, saturable and of very high affinity (K_D estimated at less than 10^{-10} M). Native cholera toxin and choleragenoid compete equally well with [^{125}I]-labeled toxin for membrane receptors. The maximum number of binding sites per cell (about 50,000) is low and of the same order of magnitude as described for rat adipocytes (Cuatrecasas, 1973*a*). Human and rat erythrocytes also bind [^{125}I]-toxin with high affinity (Cuatrecasas, 1973*a*, and *unpublished data*), although the number of sites per cell is different than in the amphibian erythrocytes.

[^{125}I]-toxin binds with such avidity that nearly all of the molecules in the medium can be rapidly adsorbed to membranes at concentrations as low as 10^{-11} M toxin (Cuatrecasas, 1973*a*). This property is useful when it is desired to correlate the concentration of active toxin, the biological activity and the number of molecules bound per cell. At high erythrocyte concentrations ($> 10^6$/ml) and at concentrations of toxin *greater* than 10^{-11} M, the number of toxin molecules bound per cell is simply the total number of toxin molecules in the medium divided by the total number of cells, provided the membrane receptor sites have not been saturated (Fig. 3*B*). The medium radioactivity remaining unbound in panel *B* (about 20%) represents contaminants or damaged toxin which is incapable of binding to either cell membranes or ganglioside-affinity adsorbents (Cuatrecasas *et al.*, 1973).

Effect of Cholera Toxin on Some Kinetic Parameters of the Toad Erythrocyte Adenylate Cyclase

Cholera toxin markedly alters the response of adenylate cyclase to its substrate, ATP,Mg^{2+} (Fig. 4). The apparent K_m is increased by about twofold, from 1.8 mM to 3.4 mM[1]. The stimulation by cholera toxin increases with increasing ATP concentration, from 20% at 0.2 mM ATP to 500% at high concentrations. 5′GTP has little effect on untreated adenylate cyclase, but it alters strikingly the toxin-modified enzyme (Fig. 4). In the presence of the guanyl nucleotide, the apparent K_m of the toxin-treated enzyme for ATP,Mg^{2+} is returned to about 2 mM, and the Hill coefficient[2] is reduced from 1.4 to 0.9. GTP elevates the velocity of the toxin-treated adenylate cyclase two- to fourfold at low ATP concentrations (< 1 mM), but has little effect at high levels of the substrate.

1 The K_m values were estimated from Hill plots. The V_{max} was obtained from a double-reciprocal plot of the data in Fig. 4.

2 Hill plots and other kinetic analyses based on the assumption of linear enzyme kinetics are used in this study only as a means of comparing untreated and toxin-activated membranes. No formal interpretations are warranted due to the accelerating phase in the time course of cyclic AMP production.

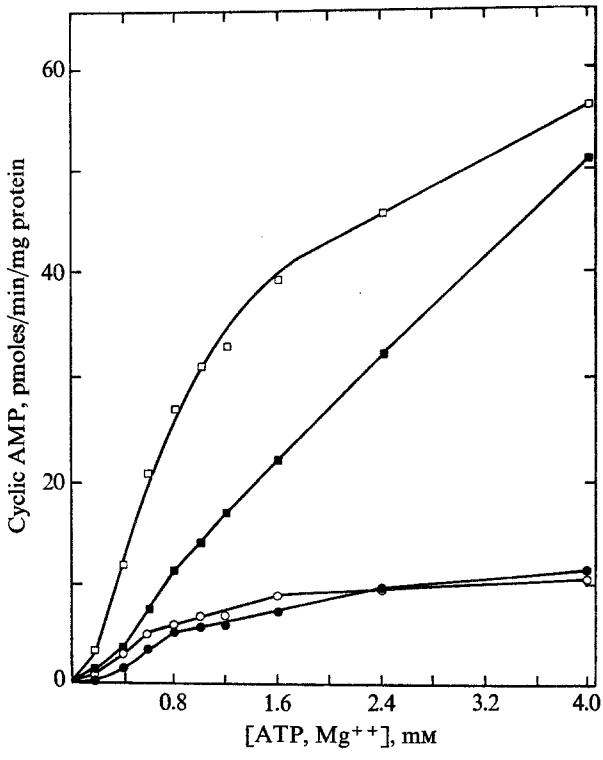

Fig. 4. The effect of increasing concentrations of ATP, Mg^{2+} in the presence (○, □) and absence (●, ■) of GTP, Mg^{2+} on the adenylate cyclase activity of control (●, ○) and cholera toxin-treated (■, □) toad erythrocyte plasma membranes. Erythrocytes were washed and incubated in the presence and absence of cholera toxin (0.3 μg/ml) as in Fig. 1. Plasma membrane adenylate cyclase activity was measured (15 min, 30 °C) in the presence of $MgCl_2$ (5 mM), [α-^{32}P]-ATP (5.4×10^6 cpm), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), 210–230 μg of membrane protein, and increasing concentrations of ATP, Mg^{2+}. Some samples (○, □) also contained GTP,Mg^{2+} (0.2 mM)

Effects of GTP on Adenylate Cyclase Activation by Cholera Toxin, (−)-Epinephrine and Sodium Fluoride

Assays were conducted at low concentrations of ATP (0.6 mM) since the GTP effects are most prominent in this range (Fig. 4). GTP stimulates adenylate cyclase if it is activated by cholera toxin or (−)-epinephrine, but it has little effect on the unstimulated enzyme or in the presence of NaF (Fig. 5). Toxin treatment and (−)-epinephrine increase the sensitivity of adenylate cyclase to GTP to nearly the same extent, and the nucleotide exhibits an identical apparent K_a (5–10 μM) in both cases. The combination of GTP and cholera toxin has a synergistic effect on the response of adenylate cyclase to catecholamines. (−)-Epinephrine stimulates the untreated ad-

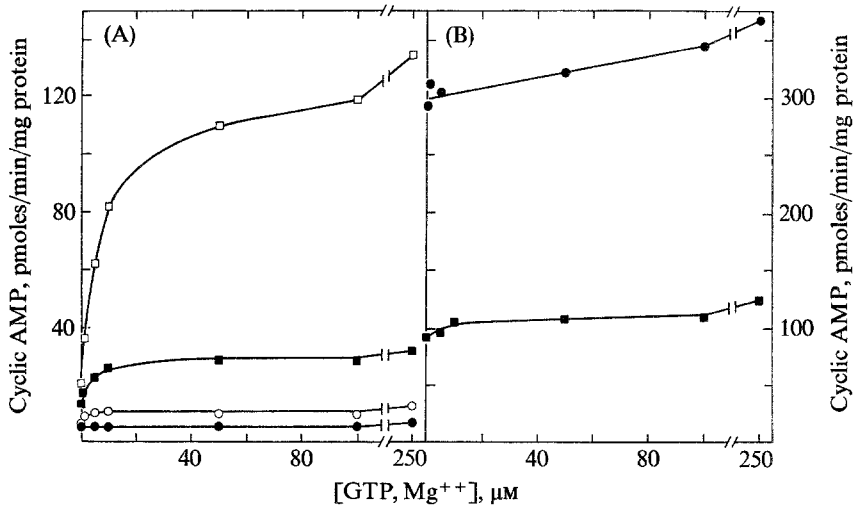

Fig. 5. Effect of increasing the concentration of GTP,Mg^{2+} on the adenylate cyclase activity of control (●, ○) and cholera toxin-treated toad erythrocyte (■, □) plasma membranes assayed in the presence (○, □) and absence (●, ■) of 20 μM (−)-epinephrine (*A*), and in the presence of 10 mM sodium fluoride (*B*). Erythrocytes were incubated in the presence and absence of cholera toxin (0.25 μg/ml) as described in Fig. 1. The plasma membrane adenylate cyclase activity was determined (15 min, 30 °C) in the presence of $MgCl_2$ (6.2 mM), [α-^{32}P]-ATP (0.5 mM, 96 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), 200–210 μg of membrane protein, and increasing concentrations of GTP, Mg^{2+}

enylate cyclase activity twofold in the presence of GTP and the activity of the toxin-treated enzyme by 60%. However, the combination of toxin treatment and GTP results in a sevenfold activation by the hormone (Fig. 5). This increased sensitivity to catecholamines is predominantly due to elevation of the maximal extent of stimulation rather than to a change in the hormone affinity (Fig. 8). The effects of GTP are not explained by an alteration in the time course of the enzymes, since the lag period is still observed in the presence of this nucleotide.

GTP has little effect on the activity assayed in the presence of NaF. Moreover, fluoride almost abolishes the effect of GTP on the toxin-treated enzyme (Fig. 5*B*). This ion can also prevent the usual (−)-epinephrine stimulation of the toxin-modified adenylate cyclase (Table 1, Fig. 6). Other purine derivatives can also activate the enzyme in the presence of cholera toxin and hormones. 5′ITP and 5′GMP stimulate to nearly the same extent as GTP, while cGMP, guanine, and the pyrimidine nucleotides are inactive. It is possible that the activity of 5′GMP could be due to its conversion to GTP by the regenerating system.

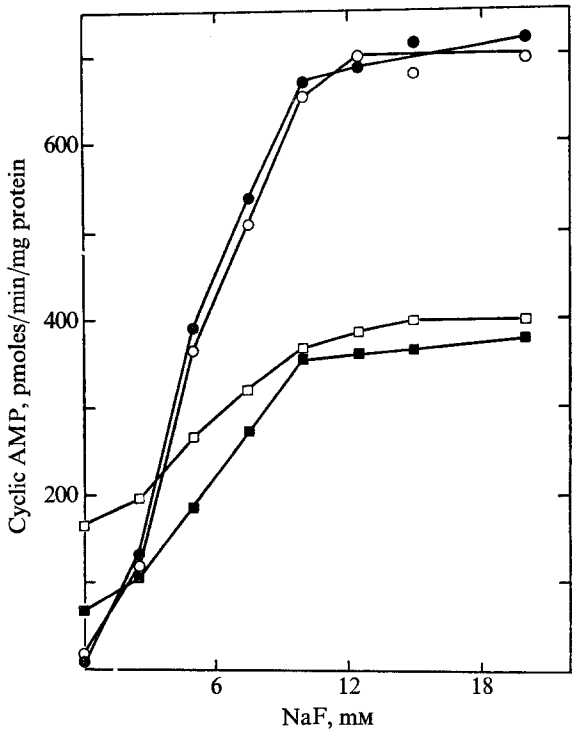

Fig. 6. The effect of increasing concentrations of sodium fluoride on the adenylate cyclase activity of control (●, ○) and cholera toxin-treated (■, □) toad erythrocyte plasma membranes assayed in the presence (○, □) and absence (●, ■) of 10 μM (−)-epinephrine. Erythrocytes were incubated in the presence and absence of cholera toxin (0.3 μg/ml) as described in Fig. 1. Plasma membrane adenylate cyclase activity was determined (15 min, 31 °C) in the presence of $MgCl_2$ (6 mM), GTP (0.2 mM), [α-^{32}P]-ATP (0.6 mM, 67 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), 110–130 μg of membrane protein, and various concentrations of NaF

Effect of Mg^{2+} on the Stimulation of Adenylate Cyclase by Cholera Toxin, (−)-Epinephrine and Sodium Fluoride

Mg^{2+} in stoichiometric excess over ATP and GTP activates the toad erythrocyte adenylate cyclase according to a simple hyperbolic relationship (Fig. 7). Both toxin and (−)-epinephrine clearly alter the response of the enzyme to magnesium. Toxin treatment lowers the apparent K_a for Mg^{2+} nearly fivefold, from 25 to 5 mM (Fig. 7*C*). (−)-Epinephrine causes nearly the same change in apparent K_a as does cholera toxin. The basal toxin-adenylate cyclase and the control enzyme activities extrapolate to similar values at infinite magnesium concentrations. Thus, although the stimulation by cholera toxin is apparent throughout, it is most prominent at low con-

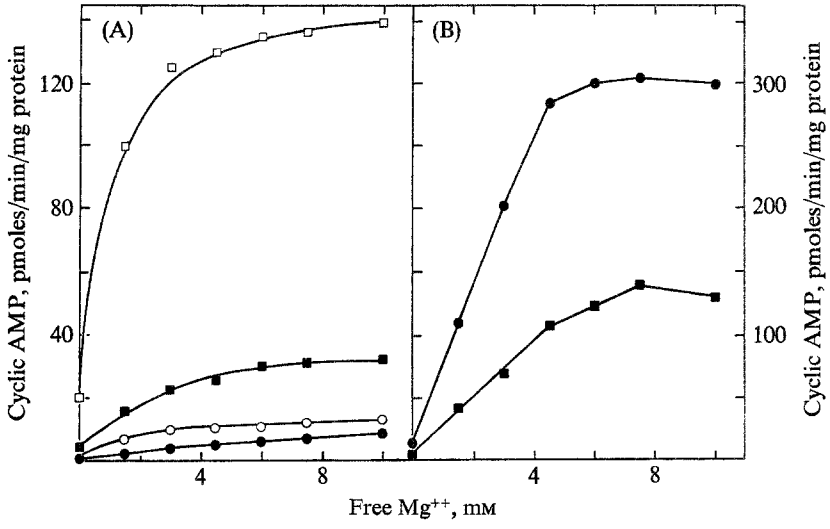

Fig. 7. The effect of increasing the concentration of free Mg^{2+} on the adenylate cyclase activity of control (●, ○) and cholera toxin-treated (■, □) toad erythrocyte plasma membranes assayed in the presence (□, ○) and absence (●, ■) of 20 μM (−)-epinephrine (*A*) and in the presence of 10 mM sodium fluoride (*B*). Erythrocytes were incubated in the presence and absence of cholera toxin (0.3 μg/ml) as in Fig. 1. The plasma membrane adenylate cyclase activity was determined (15 min, 30 °C) in the presence of $MgCl_2$ (0.6 mM), GTP, Mg^{2+} (0.2 mM), [α-^{32}P]-ATP (0.6 mM, 78 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), 220–240 μg of membrane protein, and increasing concentrations of $MgCl_2$. (*C*) Double-reciprocal plot of the data presented in (*A*) and (*B*). The activity due to Mg^{2+} was estimated by subtracting the values obtained in the absence of Mg^{2+} from the activity obtained at each concentration of magnesium

centrations of Mg^{2+}. The activity in the presence of both cholera toxin and (−)-epinephrine, however, extrapolates to a four- to fivefold higher velocity than that of either stimulant alone.

In contrast to hormones and cholera toxin, NaF stimulates adenylate cyclase by a mechanism which may be independent of changes in the affinity for magnesium (Fig. 7*B*, *C*). Furthermore, activation by the metal in the presence of F^- abruptly stops when the ratio of F^- to Mg^{2+} approaches 2:1, as has been observed previously in fat cell ghosts (Birnbaumer, Pohl & Rodbell, 1969). Sodium fluoride abolishes the toxin-induced change in the apparent K_a for free magnesium (Fig. 7*B*, *C*).

Unlike GTP, magnesium does not enhance the catecholamine stimulation $\frac{(\text{activity} + \text{hormone})}{(\text{activity} - \text{hormone})}$ of toxin-modified adenylate cyclase (Figs. 7 and 8). Both basal and hormone-stimulated activities are proportionately increased

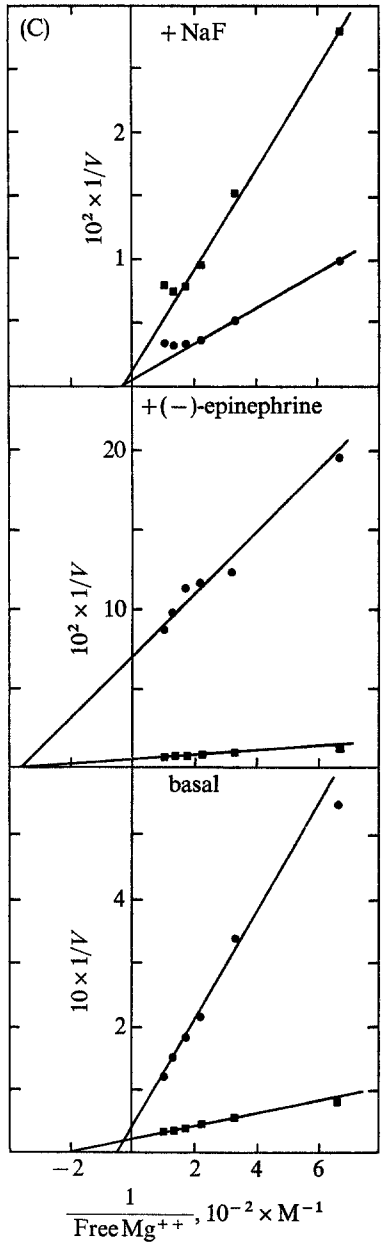

Fig. 7*C*

by free Mg^{2+} at concentrations below 10 mM (Fig. 8). The effects of GTP and magnesium on adenylate cyclase activity are interrelated in a complicated way, and are beyond the scope of this study. It is known that the apparent K_a for GTP decreases with increasing Mg^{2+} concentrations (Siegel & Cuatrecasas, 1974).

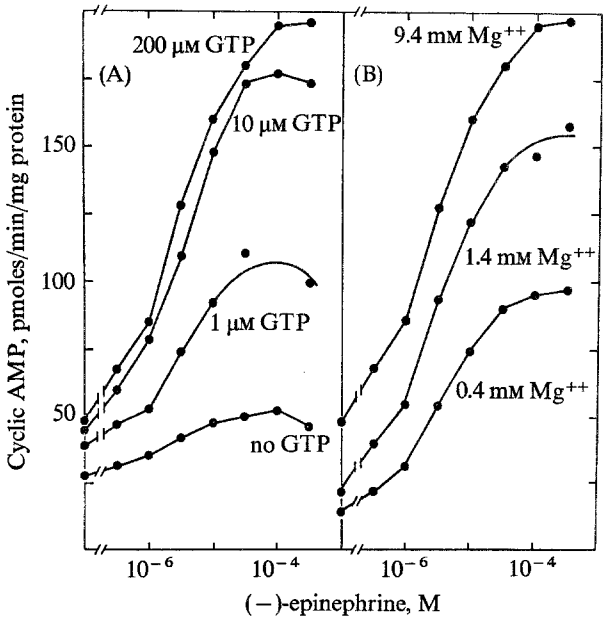

Fig. 8. Effect of GTP and free Mg^{2+} on the (−)-epinephrine stimulation of adenylate cyclase activity of cholera toxin-treated toad erythrocyte plasma membranes. Erythrocytes were incubated in the presence of cholera toxin (0.2 μg/ml) as in Fig. 1. Plasma membrane adenylate cyclase activity was measured (15 min, 30 °C) in the presence of [α-^{32}P]-ATP (0.6 mM, 89 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0) and 100 μg of membrane protein. Some samples (panel *A*) also contained 10 mM $MgCl_2$ and varying concentrations of GTP, Mg^{2+}, while others (panel *B*) contained 200 μM GTP, Mg^{2+} and varying concentrations of free Mg^{2+}

Effects of Cholera Toxin on the Catecholamine Response of Toad and Rat Erythrocyte Adenylate Cyclase

The findings described above suggest similarities between activation by hormones and cholera toxin. The action of toxin, however, cannot be identical to that of hormones, since the toxin-modified enzyme of toad erythrocytes is stimulated further by catecholamines, and, in the presence of GTP, even exhibits an enhanced response.

The toxin-treated toad erythrocyte adenylate cyclase demonstrates a fivefold increase in velocity at maximal concentrations of (−)-epinephrine, while the control enzyme is stimulated only twofold (Fig. 9). The relatively refractory state of the normal adenylate cyclase is not due to inhibition by the bitartrate salt of epinephrine since (−)-isoproterenol hydrochloride also activates the toxin-enzyme preferentially. The selective stimulation observed after exposure to toxin does not result from differences in the requirement

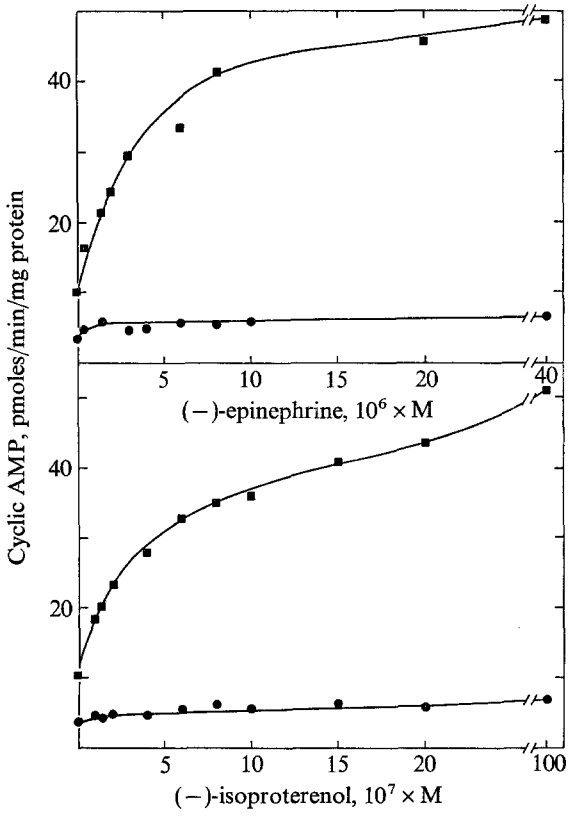

Fig. 9. Effect of increasing the concentration of (−)-epinephrine (*top*) and (−)-isoproterenol (*bottom*) on the adenylate cyclase activity of control (●) and cholera toxin-treated (■) toad erythrocyte plasma membranes. Cells were incubated in the presence and absence of cholera toxin (0.15 μg/ml) as described in Fig. 1. Plasma membrane adenylate cyclase activity was measured (15 min, 30 °C) in the presence of $MgCl_2$ (6 mM), GTP (0.2 mM), [α-^{32}P]-ATP (0.6 mM, 92 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), 80–90 μg of membrane protein, and increasing concentrations of either (−)-epinephrine or (−)-isoproterenol

for GTP or magnesium, since the concentrations of these effectors were optimal for the hormonal activation of both control and toxin-altered enzymes.

Since rat erythrocytes also contain a catecholamine-sensitive adenylate cyclase (Sheppard & Burghardt, 1969, 1970), it was of interest to determine if cholera toxin could modify the hormone response of these relatively simple, enucleate mammalian cells. Cholera toxin clearly alters adenylate cyclase activity in these cells in a way similar to that described for the toad

Table 2. Effects of cholera toxin on the adenylate cyclase activity of rat and human erythrocytes [a]

Additions	Adenylate cyclase activity [b]			
	rat, control	rat, toxin	human, control	human, toxin
None	1.7	6.5	N.D. [c]	0.04
(−)-Epinephrine, 10^{-6} M	5.8	12.1		
10^{-5} M	20.9	26.0		
10^{-4} M	29.8	34.8	0.02	0.01
Sodium fluoride, 20 mM	24.1	21.9	0.71	0.46

[a] Erythrocytes were washed in Krebs-Ringer's bicarbonate, pH 7.4, and incubated for 4 hr at 37 °C in a 1.5% suspension in the same buffer in the presence and absence of cholera toxin (0.25 μg/ml). Plasma membrane adenylate cyclase activity was assayed (15 min, 30 °C) in the presence of $MgCl_2$ (6 mM), GTP (0.2 mM), [α-^{32}P]-ATP (0.25 mM, 208 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), 90–170 μg of membrane protein, and where indicated, (−)-epinephrine or sodium fluoride (20 mM).

[b] pMoles cAMP/min/mg protein, mean values of triplicate determinations.

[c] Less than 0.01 pmoles cAMP formed per min per mg protein.

erythrocytes. The activation by NaF is inhibited, and the basal activity is stimulated and can be further activated by catecholamines (Table 2). The specific activity of the rat erythrocyte enzyme is lower than that of the toads, but it is similar to the values reported for turkey red blood cells (Bilezikian & Aurbach, 1974). Examination of the cells by phase-contrast microscopy revealed less than 1% contamination with other cell types.

Cholera toxin decreases the maximal stimulation $\frac{(\text{activity}+\text{hormone})}{(\text{activity}-\text{hormone})}$ which can be obtained with catecholamines in the rat red blood cells, while the apparent affinity for (−)-epinephrine (data not shown) and (−)-isoproterenol (Fig. 10) is increased about twofold. Choleragenoid has no effect on adenylate cyclase when incubated with these cells under conditions identical to those used with cholera toxin (Fig. 10).

Effects of Cholera Toxin on the Adenylate Cyclase Activity of Human Erythrocytes

The possibility that cholera toxin may have intrinsic adenylate cyclase was examined with human erythrocytes, which have little if any endogenous activity but bind [^{125}I]-labeled toxin with high affinity. No significant basal

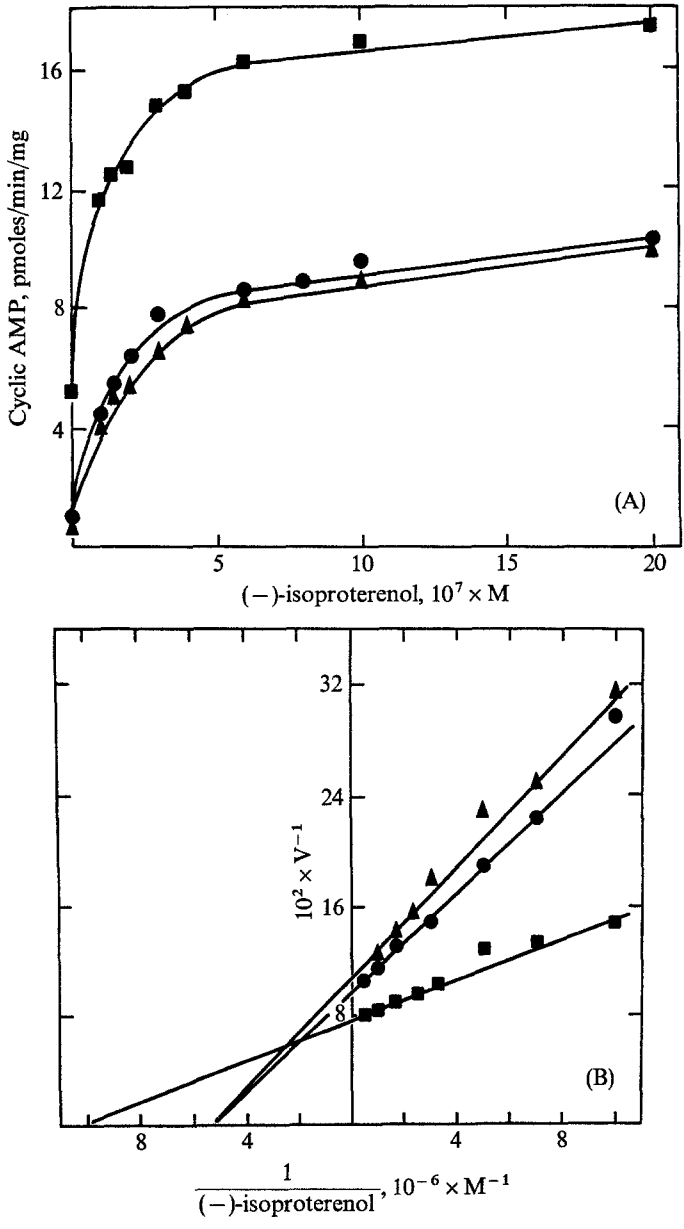

Fig. 10. (*A*) Effect of increasing the concentration of (−)-isoproterenol on the adenylate cyclase activity of control (●), choleragenoid-treated (▲) and cholera toxin-treated (■) rat erythrocytes. Rat erythrocytes were washed in Krebs-Ringer's bicarbonate, pH 7.4, and then incubated in the same buffer for 3 hr at 37 °C, either with toxin (0.28 μg/ml), choleragenoid (0.28 μg/ml) or with no additions. The plasma membrane adenylate cyclase activity was determined (12 min, 30 °C) in the presence of $MgCl_2$ (6.5 mM), [α-^{32}P]-ATP (0.27 mM, 200 cpm/pmole), GTP (0.16 mM), phosphoenolpyruvate (5.4 mM), pyruvate kinase (55 μg/ml), aminophylline (5 mM), Tris-HCl (50 mM, pH 8.0), 100 μg of membrane protein, and increasing concentrations of (−)-isoproterenol. Values are expressed as the mean of triplicate determinations. (*B*) Double-reciprocal plot of the isoproterenol-stimulated activity *vs.* isoproterenol concentration. The activity due to isoproterenol was determined by subtracting the basal values (those obtained in the absence of hormone) from the activity obtained at each concentration of isoproterenol

or catecholamine-stimulated adenylate cyclase activity is detected after incubating these cells with cholera toxin for 4 hr at 37 °C, conditions which result in a fivefold increase in activity with rat erythrocytes (Table 2). A very small quantity of adenylate cyclase activity (less than 1 pmole cAMP/min/mg) is observed with the human cells in the presence of NaF. Although this could reflect a small degree of contamination with another cell type, the possibility cannot be excluded that these erythrocytes possess a small degree of residual activity.

Discussion

The plasma membrane-bound adenylate cyclase of eukaryotic cells is modulated by hormones, purine nucleotides, and divalent metals in a complex, interrelated way (reviewed by Birnbaumer, 1973). An increase in enzyme activity can, in principle, be achieved in many ways in this multivariable system. Hormonal activation of adenylate cyclase is unique in that the stimulated enzyme exhibits a new response to purine nucleotides. 5′GTP and 5′ITP have been demonstrated to perferentially activate adenylate cyclase activity assayed in the presence of prostaglandins (Krishna, Harwood, Barber & Jamieson, 1972; Wolff & Cook, 1973), glucagon (Rodbell, Birnbaumer, Pohl & Krans, 1971), catecholamines (Leray, Chambaut & Hanoune, 1972; Bilezikian & Aurbach, 1974; Siegel & Cuatrecasas, 1974), oxytocin (Bockaert, Roy & Jard, 1972), and thyroid-stimulating hormone (Wolff & Cook, 1973). ATP at 100- to 1000-fold higher concentrations also exerts these effects (Birnbaumer, Pohl & Rodbell, 1972; Bockaert *et al.*, 1972). In contrast, NaF stimulation apparently occurs independently of and is unaffected by these nucleotides. An important objective of the present study was to define the regulatory parameters involved in the activation of adenylate cyclase by cholera toxin, and to relate these to the stimulation caused by hormones and NaF.

Exposure of intact toad erythrocytes to cholera toxin alters markedly the substrate-velocity relationship of adenylate cyclase (Fig. 4). The relative degree of stimulation by toxin increases with increasing ATP,Mg^{2+} concentrations. The apparent affinity for ATP of the toxin-modified enzyme is decreased about twofold and the Hill coefficient[2] is increased from 1.1 to 1.4. In the presence of GTP, stimulation appears to result from an increase in V_{max}; also, under these conditions the Hill coefficient is reduced to 0.9. GTP stimulates the toxin-treated adenylate cyclase at low ATP,Mg^{2+} concentrations, but the activation disappears at high concentrations of the substrate. The simplest explanation for these findings is that cholera toxin

converts the enzyme to a purine nucleotide-sensitive state, which is activated to an equal extent by both ATP and GTP but which has much higher affinity for the guanyl derivative. A similar interpretation has been suggested for the adenylate cyclase stimulation by glucagon in rat liver (Birnbaumer *et al.*, 1972), and by oxytocin in the amphibian bladder (Bockaert *et al.*, 1972). The stimulation by GTP is also of practical importance since it permits full expression of the toxin effect at low concentrations of substrate, and thus at a higher specific activity of [α-^{32}P]ATP.

The dependence of cholera toxin activation on purine nucleotides is very similar to previous findings with hormones. This suggests the possibility that the toxin might also alter adenylate cyclase activation by divalent metal ions, as has been observed with ACTH in rat fat cell ghosts (Birnbaumer *et al.*, 1969). Magnesium complexes with ATP (K_D, 10^{-4} M) to form the substrate, ATP,Mg^{2+} (Birnbaumer *et al.*, 1969; Drummond & Duncan, 1970), and at concentrations in excess of ATP it activates adenylate cyclase (Birnbaumer *et al.*, 1969; Drummond & Duncan, 1970; Bockaert *et al.*, 1972; Severson, Drummond & Sulakhe, 1972). Previous studies with metals in adenylate cyclase systems have, with few exceptions (Flawia & Torres, 1972*a*, *b*; Siegel & Cuatrecasas, 1974), ignored the well known metal chelating capacity of nucleoside triphosphates (reviewed by Izatt, Christensen & Rytting, 1971). Thus, the important variable is not the concentration of the total but the uncomplexed magnesium. Magnesium in stoichiometric excess over GTP and ATP activates the toad erythrocyte adenylate cyclase according to a simple, hyperbolic relationship (Fig. 7). Cholera toxin decreases the apparent K_a for magnesium fivefold, from 25 to 5 mM.

The cholera toxin-induced changes in the response of adenylate cyclase to GTP and magnesium are the first demonstrations that activation by the toxin involves a critical alteration in the kinetic properties of this enzyme. In pioneering work identifying adenylate cyclase as the site of action of cholera toxin, no changes in enzyme properties were observed (Kimberg *et al.*, 1971; Sharp & Hynie, 1971; Chen, Rhode & Sharp, 1972; Sharp *et al.*, 1973). These studies were performed with homogenates of intestinal mucosal cells suspended in Tris-HCl containing 12.5–25 mM $MgCl_2$. Perhaps these high concentrations of magnesium masked the increased affinity for this cation. Earlier studies also did not report effects of cholera toxin on the affinity of the enzyme for substrate. These experiments, however, were conducted with a fixed level of Mg^{2+} (5 mM in Sharp *et al.*, 1973) and with ATP concentrations up to 3 mM. Thus, it is possible that as the ATP concentrations approached those required for preferential activation of the

toxin-enzyme, the level of free Mg^{2+} was falling. The fall in unbound metal probably prevented the expected stimulation by ATP.

Sodium fluoride causes a massive, 70- to 100-fold stimulation of the toad erythrocyte adenylate cyclase, but it has no effect on the apparent affinity for magnesium and does not appreciably alter the response of the enzyme to GTP. These findings alone might suggest that F^- affects an enzyme which is different from that stimulated by hormones and cholera toxin. However, NaF also blocks the effect of the toxin on affinity for magnesium, and it prevents the usual GTP and catecholamine activation. Conversely, toxin depresses the activation by NaF; in the presence of this anion, the usual fivefold stimulation becomes a 30–60% inhibition of activity. An analogous inhibitory relationship between F^- and hormone stimulation in rat fat cell ghosts has recently been reported (Harwood & Rodbell, 1973). These findings suggest that the activation of adenylate cyclase by NaF and cholera toxin occur through different mechanisms but most likely involve the same enzyme. The mechanism of F^- stimulation involves more complex processes than just reversing "natural" inhibition of enzyme activity (Schramm & Naim, 1970), or altering a "catalytic" subunit of this enzyme (Rodbell, Birnbaumer & Pohl, 1970), since this ion also modifies the regulation by nucleotides and magnesium (but in a negative fashion). Recently, Constantopoulos and Najjar (1973) suggested that F^- activation may involve a specific dephosphorylation reaction of adenylate cyclase.

Important similarities exist between the activation of adenylate cyclase by catecholamine hormones and by cholera toxin. The extent of stimulation by GTP and the apparent K_a for this nucleotide are nearly identical in the presence of (−)-epinephrine as with cholera toxin treatment. The hormone also lowers the apparent K_a for free Mg^{2+}, and the effect is similar to that observed with toxin activation. The action of toxin is not identical to that of hormones, since the toxin-activated enzyme can be stimulated further by (−)-epinephrine, and even exhibits an accentuated catecholamine response in the presence of GTP.

The ability of cholera toxin to facilitate hormone stimulation is unusual and could be of importance in understanding the processes involved in the hormonal regulation of adenylate cyclase. The principal effect of cholera toxin on the amphibian erythrocyte enzyme appears to be an increase in the maximal degree of stimulation by catecholamines. Rat erythrocyte adenylate cyclase is also altered by cholera toxin, but in this mammalian system it is the apparent affinity for the hormone that is primarily affected (increased). These cholera toxin effects in mammalian tissues, however, are not restricted

to the response to catecholamines since a similar increase in the apparent affinity has been observed for activation of fat cell adenylate cyclase by ACTH, VIP and glucagon (unpublished observation).

The changes in the activation of adenylate cyclase by hormones could occur by modifications in the binding of the hormone with receptors, or by changes in subsequent processes. It is difficult to rationalize direct perturbations of specific hormone-receptor binding since similar alterations are observed in the responses to several chemically distinct hormones, since the maximal stimulation as well as apparent affinity for these agents are altered, since occupation of the toxin receptors by the addition of this protein directly to the membranes before assay has no effects, and since choleragenoid, which binds to the same receptors as cholera toxin (Cuatrecasas, 1973*d*), has no effects even if incubated for 4 hr with the intact cells before assay. It is suggested, therefore, that the toxin facilitates hormone stimulation by modifying processes following formation of the initial hormone-receptor complex. This could occur, for example, by increasing the rate or efficiency of adenylate cyclase activation by the hormone-receptor complex, or by stabilizing an active configuration of the enzyme, perhaps in a form more favorable for interaction with the hormone-receptor complex. These kinds of effects can be visualized within the framework of a recently suggested, two-step mechanism of hormonal activation of adenylate cyclase (Cuatrecasas, 1974*a*, *b*). In this general hypothesis, the hormone-receptor complex, once formed, must subsequently encounter and interact with entirely separate adenylate cyclase molecules by a second process which involves lateral diffusion of the interacting components within the plane of the membrane. Hormonal activation can thus be modified by processes which affect the diffusional properties of the components or by factors (e.g., possibly GTP) which stabilize the interaction between the hormone-receptor complex and adenylate cyclase. If the latter interaction is a limiting process in enzyme activation, its stabilization will not only result in enhanced enzyme activation but may also increase the *apparent* affinity of the hormone-receptor complex if this is measured only for the overall process. Most of the observed effects of the toxin could be explained if this protein were to transform adenylate cyclase into a form which is intrinsically more active and which can form more stable complexes with any one of a variety of hormone-receptor complexes.

It is interesting that a bacterial product can very specifically mimic the effects of catecholamines and potentiate their action, especially in view of the exquisite specificity usually observed in hormone-regulated systems. In understanding the possible mechanisms involved, it is pertinent that the

subunit (MW 36,000) which confers biological activity to the cholera toxin molecule (Cuatrecasas *et al.*, 1973; Finkelstein *et al.*, 1974) has strongly hydrophobic properties *(unpublished data)*, and that at least a portion of the iodinated toxin becomes irreversibly adsorbed to the cell membrane (Cuatrecasas, 1973*c*). The biological effects of the toxin are extremely protracted; these have been observed 7 to 13 days after a short period (10 min) of exposure to cultured melanoma cells (O'Keefe & Cuatrecasas, 1974). It is proposed that following binding of the native toxin molecule to gangliosides on the cell surface, a specific time and temperature-dependent process occurs within the membrane which results in the incorporation of the toxin, or a portion of the molecule (e.g., 36,000 MW subunit), in the phospholipid bilayer, thus becoming an "integral" (Singer & Nicolson, 1972), or "permanent" component of that membrane. These processes, which may involve lateral diffusion of the initial toxin-ganglioside complex or of the membrane-integrated toxin within the plane of the membrane, may ultimately result in the direct, very high affinity association of the toxin with adenylate cyclase molecules (Bennett, O'Keefe & Cuatrecasas, 1975). The toxin within the membrane may thus resemble a hormone receptor, except that it can associate with the enzyme and therefore function in the absence of complexed "hormone".

The above considerations help to explain the permanence of the toxin effects and the ability of the toxin to ubiquitously activate an enzyme normally modulated by hormones. In several systems, the toxin is demonstrably bound to the cell membrane for as long (many days, in some cases) as the biological effects are evident *(unpublished data)*. As expected from processes dependent on the diffusion of membrane components (Frye & Edidin, 1970), the action of the toxin is highly dependent on temperature and does not occur below certain critical temperatures required for phospholipid "fluidity" (Bennett & Cuatrecasas, 1975). This, and the relation of the absolute lag or latency phase in the action of the toxin to the mechanisms considered above, are described in the accompanying paper.

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Addendum. Several reports have appeared since the preparation of this manuscript which are relevant to the present findings. Field (1974) has reported that cholera toxin increases both the maximal extent and affinity of adenylate cyclase stimulation by catecholamines in turkey erythrocytes and that cholera toxin also inhibits the stimulation

of the enzyme by NaF. An increased sensitivity to catecholamine stimulation has been reported for toxin-treated liver membranes (Beckman, Flores, Witkum & Sharp, 1974), and for rat fat cells (Hewlett, Guerrant, Evans & Greenough, 1974).

References

Avruch, J., Wallach, D. F. H. 1971. Preparation and properties of plasma membrane and endoplasmic reticulum fragments from isolated rat fat cells. *Biochim. Biophys. Acta* **233**:334

Beckman, B., Flores, J., Witkum, P. A., Sharp, G. W. G. 1974. Studies on the mode of action of cholera toxin. Effects on solubilized adenyl cyclase. *J. Clin. Invest.* **53**:1202

Bennett, V., Cuatrecasas, P. 1974. Properties of the cholera toxin-stimulated adenylate cyclase of toad and rat erythrocyte plasma membranes and the mechanism of action of cholera toxin. *Fed. Proc.* **33**:1357 (Abstr.)

Bennett, V., Cuatrecasas, P. 1975. Mechanism of activation of adenylate cyclase by *Vibrio cholerae* enterotoxin. *J. Membrane Biol. 22*:29

Bennett, V., O'Keefe, E., Cuatrecasas, P. 1975. The mechanism of action of cholera toxin and the mobile receptor theory of hormone receptor-adenylate cyclase interactions. *Proc. Nat. Acad. Sci. 72*:33

Bilezikian, J. P., Aurbach, G. D. 1974. The effects of nucleotides on the expression of β-adrenergic adenylate cyclase activity in membranes from turkey erythrocytes. *J. Biol. Chem.* **249**:157

Birnbaumer, L. 1973. Hormone-sensitive adenylyl cyclases. Useful models for studying hormone receptor functions in cell-free systems. *Biochim. Biophys. Acta* **300**:129

Birnbaumer, L., Pohl, S. L., Rodbell, M. 1969. Adenyl cyclase in fat cells. I. Properties and the effects of adrenocorticotropin and fluoride. *J. Biol. Chem.* **244**:3468

Birnbaumer, L., Pohl, S. L., Rodbell, M. 1972. The glucagon sensitive adenyl cyclase system in plasma membranes of rat liver. *J. Biol. Chem.* **247**:2038

Bockaert, J., Roy, C., Jard, S. 1972. Oxytocin-sensitive adenylate cyclase in frog bladder epitheial cells. *J. Biol. Chem.* **247**:7073

Bourne, H. R., Lehrer, R. I., Lichtenstein, L. M., Weissmann, G., Zurier, R. 1973. Effects of cholera enterotoxin on adenosine 3′,5′-monophosphate and neutrophil function. Comparison with other compounds which stimulate leukocyte adenyl cyclase. *J. Clin. Invest.* **52**:698

Boyle, J. M., Gardner, J. D. 1974. Sequence of events mediating the effects of cholera toxin on rat thymocytes. *J. Clin. Invest.* **53**:1149

Bray, G. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analyt. Biochem.* **1**:279

Chen, L. C., Rohde, J. E., Sharp, G. W. G. 1972. Properties of adenyl cyclase from human jejunal mucosa during naturally acquired cholera and convalescence. *J. Clin. Invest.* **51**:731

Constantopoulos, A., Najjar, V. 1973. The activation of adenylate cyclase. II. The postulated presence of (A) Adenylate cyclase in phospho (inhibited) form. (B) A dephospho (activated) form with a cyclic adenylate stimulated membrane protein kinase. *Biochem. Biophys. Res. Commun.* **53**:794

Cuatrecasas, P. 1971. Insulin-receptor interactions in adipose tissue cells: Direct measurement and properties. *Proc. Nat. Acad. Sci.* **68**:1264

Cuatrecasas, P. 1973*a*. The interaction of *Vibrio cholerae* enterotoxin with cell membranes. *Biochemistry* **12**:3547

Cuatrecasas, P. 1973*b*. Gangliosides and membrane receptors for cholera toxin. *Biochemistry* **12**:3558

Cuatrecasas, P. 1973*c*. Cholera toxin-fat cell interaction and the mechanism of activation of the lipolytic response. *Biochemistry* **12**:3567

Cuatrecasas, P. 1973*d*. *Vibrio cholerae* choleragenoid—Mechanism of inhibition of cholera toxin action. *Biochemistry* **12**:3577

Cuatrecasas, P. 1974*a*. Membrane receptors. *Annu. Rev. Biochem.* **43**:169

Cuatrecasas, P. 1974*b*. Insulin receptors, cell membranes and hormone action. *Biochem. Pharmacol.* **23**:2353

Cuatrecasas, P., Parikh, I., Hollenberg, M. D. 1973. Affinity chromatography and structural analysis of *Vibrio cholerae* enterotoxin—Ganglioside agarose and the biological effects of ganglioside-containing soluble polymers. *Biochemistry* **12**:4253

Donta, S. T., King, M., Sloper, K. 1973. Induction of steroidogenesis in tissue culture by cholera enterotoxin. *Nature, New Biol.* **243**:246

Drummond, G. I., Duncan, L. 1970. Adenyl cyclase in cardiac tissue. *J. Biol. Chem.* **245**:976

Evans, D. J., Chen, L. C., Carlin, G. T., Evans, D. G. 1972. Stimulation of adenyl cyclase by *Escherichia coli* enterotoxin. *Nature, New Biol.* **236**:137

Field, M. 1974. Mode of action of cholera toxin: Stabilization of catecholamine-sensitive adenylate cyclase in turkey erythrocytes. *Proc. Nat. Acad. Sci* **71**:3299

Finkelstein, R. A. 1973. Cholera. *CRC Crit. Rev. Microbiol.* **2**:553

Finkelstein, R. A., Boesman, M., Neoh, S. H., La Rue, M. K., Delaney, R. 1974. Dissociation and recombination of the subunits of the cholera enterotoxin (choleragen). *J. Immunol.* **113**:145

Finkelstein, R. A., LoSpalluto, J. J. 1969. Pathogenesis of experimental cholera. Preparation and isolation of choleragen and choleragenoid. *J. Exp. Med.* **130**:185

Finkelstein, R. A., LoSpalluto, J. J. 1970. Production of highly purified choleragen and choleragenoid. *J. Infect. Dis.* **121**:S63

Flawia, M. M., Torres, H. N. 1972*a*. Adenylate cyclase activity in *Neurospora crassa*. I. General properties. *J. Biol. Chem.* **247**:6873

Flawia, M. M., Torres, H. N. 1972*b*. Adenylate cyclase activity in *Neurospora crassa*. II. Kinetics. *J. Biol. Chem.* **247**:6880

Frye, C. D., Edidin, M. 1970. The rapid intermixing of cell surface antigens after formation of mouse-human heterokargons. *J. Cell Sci.* **7**:313

Glynn, I. M., Chappell, J. B. 1964. A simple method for the preparation of ^{32}P-labelled adenosine triphosphate of high specific activity. *Biochem. J.* **90**:147

Gorman, R. E., Bitensky, M. W. 1972. Selective effects of cholera toxin on the adrenaline responsive component of hepatic adenyl cyclase. *Nature* **235**:439

Harwood, J. P., Rodbell, M. 1973. Inhibition by fluoride ion of hormonal activation of fat cell adenylate cyclase. *J. Biol. Chem.* **248**:4901

Hewlett, E. L., Guerrant, R. L., Evans, D. J., Greenough, W. B. 1974. Toxins of *Vibrio cholerae* and *Escherichia coli* stimulate adenyl cyclase in rat fat cells. *Nature, New Biol.* **249**:371

Hollenberg, M. D., Fishman, P. H., Bennett, V., Cuatrecasas, P. 1974. Cholera toxin and cell growth: Role of membrane gangliosides. *Proc. Nat. Acad. Sci.* **71**:4224

Holmgren, J., Lindholm, L., Lonnroth, I. 1974. Interaction of cholera toxin and toxin derivatives with lymphocytes. *J. Exp. Med.* **139**:801

Holmgren, J., Lonnroth, I., Ouchterlony, O., Svennerholm, A.-M. 1972. Studies on cholera toxins. *J. Gen. Microbiol.* **73**:XXIX

Holmgren, J., Lonnroth, I., Svennerholm, L. 1973. Tissue receptor for cholera exotoxin: Postulated structure from studies with G_{M1} ganglioside and related glycolipids. *Infect. Immun.* **8**:208

Hunter, W. M., Greenwood, F. C. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* **194**:495

Izatt, R. M., Christensen, J. J., Rytting, J. H. 1971. Sites and thermodynamic quantities associated with proton and metal ion interaction with ribonucleic acid, deoxyribonucleic acid and their constituent bases, nucleosides and nucleotides. *Chem. Rev.* **71**:439

Kimberg, D. V., Field, M., Johnson, J., Henderson, A., Gershon, E. 1971. Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. *J. Clin. Invest.* **50**:1218

King, C. A., van Heyningen, W. E. 1973. Inactivation of cholera toxin by sialidase-resistant monosialoganglioside. *J. Infect. Dis.* **127**:639

Krishna, G., Harwood, J., Barber, A., Jamieson, G. A. 1972. Requirement for guanosine triphosphate in the prostaglandin activation of adenylate cyclase of platelet membrane. *J. Biol. Chem.* **247**:2253

Leray, F., Chambaut, A.-M., Hanoune, J. 1972. Adenylate cyclase activity of rat-liver plasma membranes. Hormonal stimulations and effect of adrenalectomy. *Biochem. Biophys. Res. Commun.* **48**:1385

Lonnroth, I., Holmgren, J. 1973. Subunit structure of cholera toxin. *J. Gen. Microbiol.* **76**:417

LoSpalluto, J. J., Finkelstein, R. A. 1972. Chemical and physical properties of cholera exo-enterotoxin (choleragen) and its spontaneously formed toxoid (choleragenoid). *Biochim. Biophys. Acta* **257**:158

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the folin-phenol reagent. *J. Biol. Chem.* **193**:265

Mashiter, K., Mashiter, G. D., Hauger, R. L., Field, J. B. 1973. Effects of cholera and *E. coli* enterotoxins on cyclic adenosine 3′,5′-monophosphate levels and intermediary metabolism in the thyroid. *Endocrinology* **92**:541

Mosley, W. H., Ahmed, A. 1969. Active and passive immunization in the adult rabbit ileal loop model as an assay for production of antitoxin immunity by cholera vaccines. *J. Bacteriol.* **100**:547

O'Keefe, E., Cuatrecasas, P. 1974. Cholera toxin mimics melanocyte stimulating hormone in inducing differentiation in melanoma cells. *Proc. Nat. Acad. Sci.* **71**:2500

Pierce, N. F. 1973. Differential inhibitory effects of cholera toxoids and ganglioside on the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. *J. Exp. Med.* **137**:1009

Ramachandran, J. 1971. A new simple method for separation of adenosine 3′,5′-cyclic monophosphate from other nucleotides and its use in the assay of adenyl cyclase. *Analyt. Biochem.* **43**:227

Rodbell, M., Birnbaumer, L., Pohl, S. L. 1970. Adenyl cyclase in fat cells. Stimulation by secretin and the effects of trypsin on the receptors for lipolytic hormones. *J. Biol. Chem.* **245**:718

Rodbell, M., Birnbaumer, L., Pohl, S. L., Krans, H. M. 1971. The glucagon-sensitive adenyl cyclase system in plasma membrane of rat liver. *J. Biol. Chem.* **246**:1877

Rodbell, M., Lin, M. C., Salamon, Y. 1974. Evidence for independent action of glucagon and nucleotides on the hepatic adenylate cyclase system. *J. Biol. Chem.* **249**:59

Rosen, O. M., Rosen, S. M. 1968. The effect of catecholamines on adenyl cyclase of frog and tadpole hemolysates. *Biochem. Biophys. Res. Commun.* **31**:82

Rosen, O. M., Rosen, S. M. 1969. Properties of an adenyl cyclase partially purified from frog erythrocytes. *Arch. Biochem. Biophys.* **131**:449

Schramm, M., Naim, E. 1970. Adenyl cyclase of rat parotid gland. *J. Biol. Chem.* **245**:3225

Severson, D. L., Drummond, G. I., Sulakhe, P. V. 1972. Adenylate cyclase in skeletal muscle. Kinetic properties and hormonal stimulation. *J. Biol. Chem.* **247**:2949

Sharp, G. W. G. 1973. Action of cholera toxin on fluid and electrolyte movement in the small intestine. *Annu. Rev. Med.* **24**:19

Sharp, G. W. G., Hynie, S. 1971. Stimulation of intestinal adenyl cyclase by cholera toxin. *Nature* **229**:266

Sharp, G. W. G., Hynie, S., Ebel, H., Parkinson, D. K., Witkum, P. 1973. Properties of adenyl cyclase in mucosa cells of the rabbit ileum and the effect of cholera toxin. *Biochim. Biophys. Acta* **309**:339

Sheppard, H., Burghardt, C. 1969. Adenyl cyclase in non-nucleated erythrocytes of several mammalian species. *Biochem. Pharmacol.* **18**:2576

Sheppard, H., Burghardt, C. 1970. The stimulation of adenyl cyclase of rat erythrocyte ghosts. *Molec. Pharmacol.* **6**:425

Siegel, M. I., Cuatrecasas, P. 1974. Epinephrine stimulation of fat cell adenylate cyclase: Regulation by guanosine-5′-triphosphate and magnesium ion. *Molec. Cell. Endocrinol.* **1**:89

Singer, S. J., Nicolson, G. L. 1972. The fluid mosaic model of the structure of cell membranes. *Science* **175**:720

Symons, R. H. 1968. Modified procedure for the synthesis of ^{32}P-labelled ribonucleoside 5′-monophosphate of high specific activity. *Biochim. Biophys. Acta* **155**:609

van Heyningen, S. 1974. Cholera toxin: Interaction of subunits with ganglioside G_{M1}. *Science* **183**:656

van Heyningen, W. E. 1973. A note on the specific fixation, specific deactivation and non-specific inactivation of bacterial toxins by gangliosides. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **276**:297

van Heyningen, W. E., Carpenter, W. B., Pierce, N. F., Greenough, W. B. III. 1971. Deactivation of cholera toxin by ganglioside. *J. Infect. Dis.* **124**:415

van Heyningen, W. E., Mellanby, J. 1968. The effect of cerebroside and other lipids on the fixation of tetanus toxin by gangliosides. *J. Gen. Microbiol.* **52**:447

van Heyningen, W. E., Miller, P. A. 1961. The fixation of tetanus toxin by gangliosides. *J. Gen. Microbiol.* **24**:107

Vaughan, M., Pierce, N. F., Greenough, W. B. III. 1970. Stimulation of glycerol production in fat cells by cholera toxin. *Nature* **226**:658

Walker, W. A., Field, M., Isselbacher, K. J. 1974. Specific binding of cholera toxin to isolated intestinal microvillus membranes. *Proc. Nat. Acad. Sci.* **71**:320

White, A. A., Zenser, T. V. 1971. Separation of cyclic 3′,5′-nucleoside monophosphate from other nucleotides on aluminum oxide columns application to the assay of adenyl cyclase and guanyl cyclase. *Analyt. Biochem.* **41**:372

Wolff, J., Cook, G. H. 1973. Activation of thyroid membrane adenylate cyclase by purini nucleotides. *J. Biol. Chem.* **248**:350

Wolff, J., Temple, R., Cook, G. H. 1973. Stimulation of steroid secretion in adrenal tumor cells by choleragen. *Proc. Nat. Acad. Sci.* **70**:2741