Proc. Nat. Acad. Sci. USA
Vol. 71, No. 8, pp. 3299–3303, August 1974

Mode of Action of Cholera Toxin: Stabilization of Catecholamine-Sensitive Adenylate Cyclase in Turkey Erythrocytes

(cyclic AMP/epinephrine/hormone receptor/fluoride)

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Communicated by Kurt J. Isselbacher, June 3, 1974

ABSTRACT **Preincubating turkey erythrocytes with cholera toxin alters their adenylate cyclase (EC 4.6.1.1) system: basal activity, maximal epinephrine-stimulatable activity, and affinity of the enzyme reaction for epinephrine are all increased. Pretreatment of erythrocytes with choleragenoid prevents these changes. Cholera toxin does not alter [^{3}H]epinephrine uptake by intact erythrocytes. The increase in epinephrine-stimulatable cyclase activity appears to occur at the expense of fluoride-stimulatable activity, which is decreased by the toxin. In lysates from both toxin-treated and control cells, maximally stimulating amounts of epinephrine and fluoride, when added in combination, have a nearly additive effect on cyclase activity. These observations suggest that the adenylate cyclase system of the turkey erythrocyte may exist in two interconvertible forms, one that is catecholamine-responsive but fluoride-insensitive, and another that is fluoride-sensitive but not coupled to catecholamine receptors. Cholera toxin appears to stabilize the enzyme in its hormone receptor-coupled form.**

Vibrio cholerae enterotoxin (hereafter called "toxin") increases adenylate cyclase (EC 4.6.1.1) activity in the small intestine, thereby stimulating a cAMP-dependent active secretory process (1). The ability of the toxin to enhance adenylate cyclase activity has been directly or indirectly demonstrated in a variety of tissues. Significant progress has been made in elucidating the structure of cholera toxin (2–6) and identifying a cell membrane receptor (7–10), but the mechanism by which the toxin, once bound to receptor, stimulates adenylate cyclase is not understood (11).

This communication describes the effects of cholera toxin on the adenylate cyclase system of the turkey erythrocyte. Heretofore this system had been found to exhibit significant enzyme activity only upon addition of β-adrenergic agonists or fluoride (12–15). Cholera toxin, if added to intact erythrocytes, increases adenylate cyclase activity and alters the kinetics of the enzyme's interactions with both epinephrine and fluoride.

MATERIALS AND METHODS

Cholera toxin (molecular weight = 84,000), purified by the method of Finkelstein and LoSpalluto (16) was provided by the SEATO Cholera Research Program and was prepared under contract for the National Institute of Allergy and Infectious Diseases by Dr. R. A. Finkelstein, the University of Texas Southwestern Medical School, Dallas, Texas. Purified choleragenoid (molecular weight = 56,000), prepared by the procedure described by Finkelstein and LoSpalluto (16), was a gift of Dr. R. A. Finkelstein. L-Epinephrine was obtained as Adrenalin chloride from Parke-Davis (Detroit, Mich.) and propranolol as Inderal from Ayerst Laboratories (New York, N.Y.). Trisodium phospho*enol*pyruvate, pyruvate kinase (type II), ATP, and cAMP were obtained from Sigma (St. Louis, Mo.). [^{3}H]cAMP (20–24 Ci/mmole), [α-^{32}P]ATP (8–10 Ci/mmole), DL-[7-^{3}H]epinephrine (9.45 Ci/mmole), and [^{14}C]sucrose (0.4 Ci/mmole) were obtained from New England Nuclear Corp. (Boston, Mass.). Merck aluminum oxide (neutral, activity I for column chromatography) was obtained from Brinkmann Instruments (Westbury, N.Y.).

Fresh turkey erythrocytes suspended in a modified (1) Krebs-Ringer-bicarbonate solution (KRB) were incubated with toxin for 10 min in the cold (0.1–0.25 ml of packed cells per 1.0 ml of KRB containing 10^{-8} to 10^{-10} M toxin), then washed twice with KRB and incubated with shaking for 3–4 hr at 38° in KRB containing 40 mM D-glucose and 0.2% bovine-serum albumin (standard medium). The suspensions (1 ml of packed cells per 2.5–4 ml of medium) were gassed with 5% CO_2 in O_2.

cAMP was determined in 0.1-ml aliquots of cell suspensions to which 5 ml of 5% (w/v) Cl_3CCOOH containing nCi [^{3}H]-cAMP as recovery marker, was added. The Cl_3CCOOH was extracted with diethyl ether and cAMP was assayed in duplicate using the protein kinase binding procedure described by Gilman (17). Results were expressed as pmoles of cAMP in the cell suspension divided by packed cell volume. The latter was determined by centrifuging at 1200 × *g* for 10 min. Incubation medium blanks (medium not exposed to cells) gave an apparent cAMP concentration of 0.026 pmoles/μl (n = 3). After correction for this, cAMP accumulation in the medium during incubation with cells at 38° for 3.5 hr was found to be 0.027 pmoles of cAMP/μl in control incubations and 0.091 pmoles/μl in toxin incubations (n = 3 for both). Comparison with values in Table 1 indicates that more than 85% of total suspension cAMP was intracellular.

To determine adenylate cyclase activity, we apportioned erythrocytes suspended in original incubation medium into individual assay tubes (100 μl of packed cells per tube) and centrifuged them for 5 min at 1000 × *g*. The cells were then hemolyzed by two additions of 0.4 ml of water followed by two additions of 0.4 ml of 50 mM Tris·HCl buffer (pH 7.4) containing 5 mM $MgCl_2$ (Tris–Mg buffer). Between additions samples were shaken and centrifuged for 5 min at 1000 × *g* and the supernates were discarded; after the final addition, samples were centrifuged for 10 min at 2000 × *g*. The final lysate contained 6.9 mg of protein and 1.5 μmoles of K^+ per 100 μl of packed cells. Phase contrast microscopy revealed intact, nuclei-containing ghosts.

Abbreviation: KRB, Krebs-Ringer-bicarbonate.

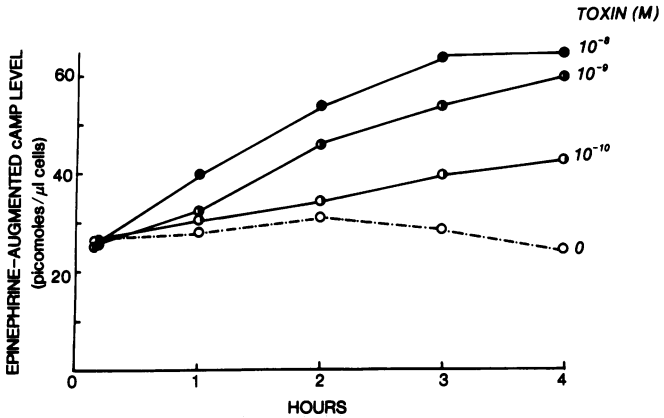

FIG. 1. Effect of cholera toxin on epinephrine-augmented cAMP levels. Erythrocytes, after having been exposed to various concentrations of toxin for 10 min at 4°, were incubated at 38° in standard medium for various times. Epinephrine (50 μM) was added 10 min before end of incubation. Each point is the mean of three separate experiments.

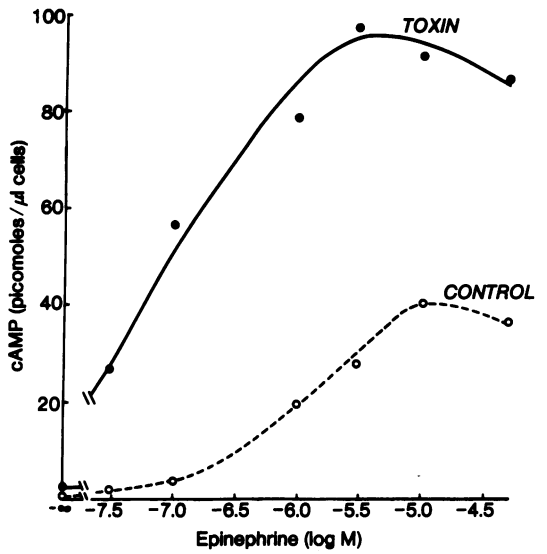

FIG. 2. Effect of cholera toxin on cAMP levels at various epinephrine concentrations. Toxin-treated (10 nM) and control cells were incubated for 3.5 hr at 38° in standard medium. Epinephrine was added 10 min before end of incubation. Each point is the mean of four separate experiments.

To each tube of lysate was added 0.2 ml of Tris–Mg buffer containing 0.15 μCi of [α-^{32}P]ATP, 0.06 μmoles of ATP, 0.3 μmoles of cAMP, 3.5 μmoles of phospho*enol*pyruvate, and 150 μg of pyruvate kinase. Final concentrations of ATP and cAMP were 0.2 mM and 1.0 mM, respectively. All steps thus far were carried out in the cold. Samples were then incubated at 37° for 20 min. [^{3}H]cAMP (0.01 μCi) was then added as recovery marker and the tubes were immediately placed in boiling water. After centrifuging, the supernates were applied to aluminum oxide columns to separate [^{32}P]-cAMP from other ^{32}P-labeled compounds (18). Each column was eluted with 2.5 ml of Tris-Mg buffer; the first 0.5 ml of effluent was discarded and the next 2.0 ml were dissolved in 10 ml of Bray's solution (19) and assayed for ^{3}H and ^{32}P in a liquid scintillation spectrometer. Recoveries of [^{3}H]cAMP were about 75%. All samples were assayed in duplicate. In each experiment, heat-denatured lysates were also assayed and their apparent activities [0.18 ± 0.026 (1 SEM) pmoles/20 min per μl of cells; n = 10] were subtracted from all other values. Adenylate cyclase activity was found to be constant with time for 30 min, proportional to packed cell volume over the range 50–200 μl, and higher in the presence of 1.0 mM cAMP than in the presence of either 0.1 or 2.5 mM cAMP.

Epinephrine uptake by intact erythrocytes was measured by adding DL-[^{3}H]epinephrine (1 μCi/ml) and various concentrations of L-epinephrine* to erythrocytes suspended in buffer (0.1 ml packed cells/ml) consisting of 150 mM NaCl, 1.25 $CaCl_2$, 1.1 mM $MgCl_2$, and 20 mM Tris·HCl, pH 7.4 (Tris–saline). After incubating with [^{3}H]epinephrine, suspensions were centrifuged in the cold for 2–5 min at 2500 × g, the supernates were removed and the cells were resuspended in 10 ml of cold Tris–saline and centrifuged again. Cells were then resuspended in 1.0 ml of Tris–saline to which 0.25 ml of 25% Cl_3CCOOH was added. After 30 min, each sample was centrifuged and the entire Cl_3CCOOH supernate was dissolved in 10 ml of Bray's solution (19) and assayed for ^{3}H in a liquid scintillation spectrometer. In some experiments [^{14}]sucrose (2 μCi/ml) was added to incubations as an extracellular marker. The cpm of ^{3}H in the buffer washes and Cl_3CCOOH supernates were then corrected for contamination with original incubation medium by the formula: $^3H_{bound} = {}^3H_{sample} - {}^{14}C_{sample} \times {}^3H_{medium}/{}^{14}C_{medium}$. The [^{14}C]-sucrose correction accounted for 70–90% of the cpm of ^{3}H in the buffer washes and 0–10% of the cpm of ^{3}H in the Cl_3-CCOOH supernates. Metabolism of bound epinephrine was evaluated by thin-layer chromatography: cells were pre-incubated with [^{3}H]epinephrine, washed twice with cold Tris-saline and extracted with 0.5 M HCl; extracts were then lyophilized, redissolved in 1 mM HCl, and chromatographed on cellulose paper in 1-butanol saturated with 5% trifluoro-acetic acid.

TABLE 1. *Effects of cholera toxin, choleragenoid, and epinephrine on cAMP levels of intact erythrocytes*

Cell treatment	pmoles of cAMP/μl of cells	
	−Epinephrine	+Epinephrine
Part A: 50 μM epinephrine		
Control	0.9 ± 0.25	29.0 ± 3.1
Cholera toxin	2.5 ± 0.37*	80.0 ± 8.0*
Part B: 0.1 μM epinephrine		
Control	—	2.1 ± 0.28
Choleragenoid	—	3.3 ± 0.44*
Cholera toxin	—	23.6 ± 4.4*
First choleragenoid, then cholera toxin	—	4.8 ± 0.76*

* Greater than control, $P < 0.02$.

Part A: Suspensions of toxin-treated and control erythrocytes were incubated for 3.5 hr at 38° in standard medium. Ten minutes before end of incubation epinephrine (50 μM) was added to some suspensions. Values are means ± 1 SEM for six experiments.

Part B: Suspensions of erythrocytes were incubated for 5 min at 4° with choleragenoid (1.5 nM), cholera toxin (0.1–1 nM), or KRB alone and then centrifuged. Cells were washed twice with KRB, resuspended, and incubated for 5 min at 4° with toxin (0.1–1 nM) or KRB alone. Cells were washed twice again and then incubated in standard medium for 3.5 hr at 38°. Epinephrine (0.1 μM) was added to suspensions 10 min before end of incubation. Values are means ± 1 SEM for four experiments.

* Turkey erythrocyte membrane receptor sites for catecholamines do not distinguish between D- and L-isomers (13, 14, 20).

Statistical analyses were by Student's t test for paired variates.

RESULTS

cAMP Levels in Intact Erythrocytes. cAMP levels, measured after a 3.5 hr incubation at 38°, are shown in Table 1, part *A*. In the absence of epinephrine there was a low but detectable level of cAMP. The level was about 3-fold greater in cells pretreated with 10 nM cholera toxin. Addition of epinephrine (to 50 μM) 10 min prior to the end of incubation increased cAMP levels 30-fold, the levels again being about 3-fold greater in toxin-treated than in control cells.

Fig. 1 shows cAMP responses to epinephrine (50 μM added 10 min before terminating incubation) at various times after exposure to cholera toxin. At each concentration of toxin tested, an increase in cAMP was apparent at 1 hr (earlier samples were not taken) and a peak effect was reached in 3 hr with 10 nM toxin.† As shown in Fig. 2, pretreatment of cells with toxin decreased the concentration of epinephrine required to achieve a half-maximal effect (hereafter signified by "K_m"). When the data summarized in Fig. 2 are plotted by the double reciprocal method (plots not shown), the calculated V_{max} for cAMP accumulation in response to epinephrine is 2.4-fold greater in toxin-treated cells [97.3 ± 4.1 (1 SEM) pmoles/μl of cells per 10 min as compared to 40.8 ± 2.2 in controls, $P < 0.001$] and the calculated K_m for epinephrine is 16-fold lower in toxin-treated cells (0.087 ± 0.018 μM as compared to 1.4 ± 0.34 μM in controls, $P < 0.02$).

Choleragenoid, a biologically inactive protein that is immunologically indistinguishable from cholera toxin (2), has been shown to block binding of the toxin to liver membranes (21) and thymocytes (22) and to block the lipolytic effect of toxin in isolated fat cells (21). As indicated in Part *B* of Table 1, choleragenoid also blocks the effect of toxin on turkey erythrocytes. Since the cells were washed to remove unbound choleragenoid before being exposed to toxin, choleragenoid presumably competes with toxin for the same membrane receptor sites and is not readily dissociated from these sites. Choleragenoid by itself slightly increased the epinephrine-augmented cAMP level.

Adenylate Cyclase Activity in Erythrocyte Lysates. In comparison to control lysates, cyclase activity in lysates of toxin-treated cells was greater under "basal" conditions (no epinephrine or fluoride added), was subject to greater enhancement by a maximally stimulating concentration of epinephrine, and had a lower K_m for epinephrine (see Table 2 and Fig. 3). These effects are qualitatively similar, therefore, to effects of toxin on cAMP levels in intact erythrocytes. Addition of propranolol (10 μM) to lysates of toxin-treated cells completely inhibited the effect of 10 μM epinephrine, but did not reduce basal cyclase activity. The latter, therefore, does not appear to be due to endogenous catecholamine.

Fluoride-stimulatable cyclase activity was substantially lower in lysates from toxin-treated cells than in control lysates. As indicated in Fig. 4, cholera toxin reduced the maximal fluoride response but did not affect its K_m (about 3.5 mM). With both toxin-treated and control lysates, maximally stimulating amounts of epinephrine and fluoride, when added in combination, had a nearly additive effect on cyclase activity (see last 2 *rows* in Table 2).

† The cholera toxin concentrations shown in Fig. 1 are those initially present in the preincubation media. Since the preincubation hematocrit was about 20%, much of the toxin may have been adsorbed onto the cells. If one assumes 100% adsorption, an initial toxin concentration of 0.1 nM would result in 60 molecules bound per cell. Thus, very few molecules are required for stimulation.

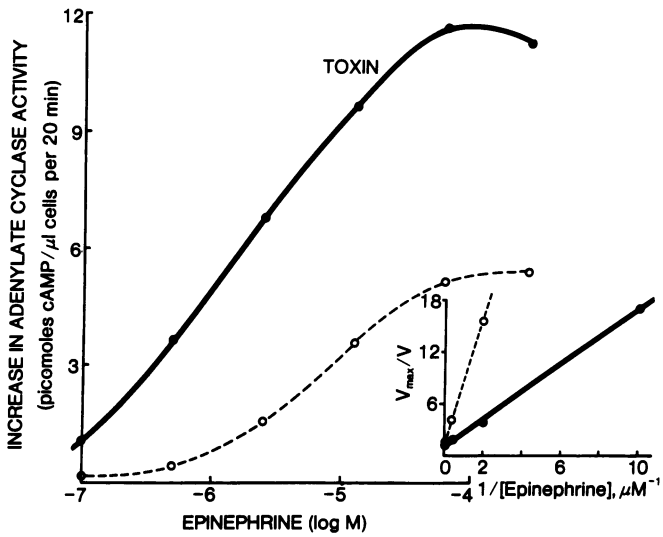

FIG. 3. Adenylate cyclase activity of lysates from toxin-treated and control cells as a function of epinephrine concentration. Toxin-treated (10 nM) and control cells were preincubated in standard medium for 3.5 hr. Lysates were then assayed at various epinephrine concentrations. Each point is the mean of seven separate experiments. The *y-axis* shows the increase in cyclase activity above the level present in the absence of epinephrine (the mean basal levels in toxin-treated and control cells were 1.53 ± 0.16 and −0.06 ± 0.08, respectively). V_{max} for epinephrine, calculated as the mean of the greatest increments in cyclase activity reached in each experiment at any epinephrine concentration, was 12.04 ± 2.07 for toxin-treated cells and 5.42 ± 1.45 for control cells. This difference is significant to $P < 0.01$. In the *insert* the same data are plotted as V_{max}/V against reciprocal of epinephrine concentration. Values for K_m (means of slopes of least square regression lines calculated from each experiment) were 1.60 ± 0.32 μM for toxin-treated cell lysates and 7.28 ± 0.83 μM for control cell lysates. The difference in K_m is significant to $P < 0.001$.

TABLE 2. *Effect of cholera toxin on basal, epinephrine-stimulated, and fluoride-stimulated adenylate cyclase activities of erythrocyte lysates*

Assay conditions	Adenylate cyclase activity (pmoles of cAMP/μl of cells per 20 min)	
	Control cells	Toxin-treated cells
A. Basal	0.07 ± 0.015	1.28 ± 0.20
B. Epinephrine (0.1 mM)	5.18 ± 0.52	11.92 ± 1.50
C. NaF (10 mM)	15.65 ± 1.42	6.28 ± 0.67
D. Epinephrine (0.1 mM) + NaF (10 mM)	18.55 ± 1.11*	16.90 ± 2.28*
(B − A) + (C − A)	20.70 ± 1.69	15.64 ± 1.69
(D − A)	18.49 ± 1.11	15.62 ± 2.20

* Greater than NaF alone and epinephrine alone, $P < 0.01$.

Toxin-treated (10 nM) and control cells were incubated in standard medium for 3.5 hr at 38°. Cells were then hemolyzed and their adenylate cyclase activity was assayed. Results are means ± 1 SEM for 10 experiments. All conditions were tested in each experiment.

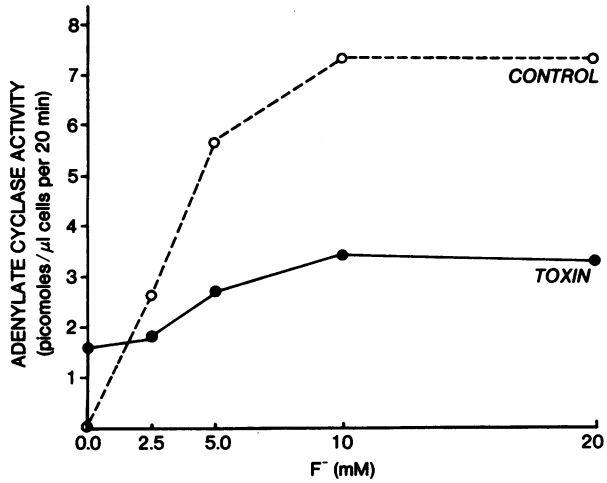

FIG. 4. Adenylate cyclase activity as a function of fluoride concentration. Each point is the mean of three separate experiments. *Toxin* refers to cells preincubated with 10 nM cholera toxin.

A preincubation period of between 30 and 60 min is required before a change in adenylate cyclase activity can be demonstrated (10 nM toxin, 38°). No change in cyclase activity can be demonstrated after preincubation of cells with

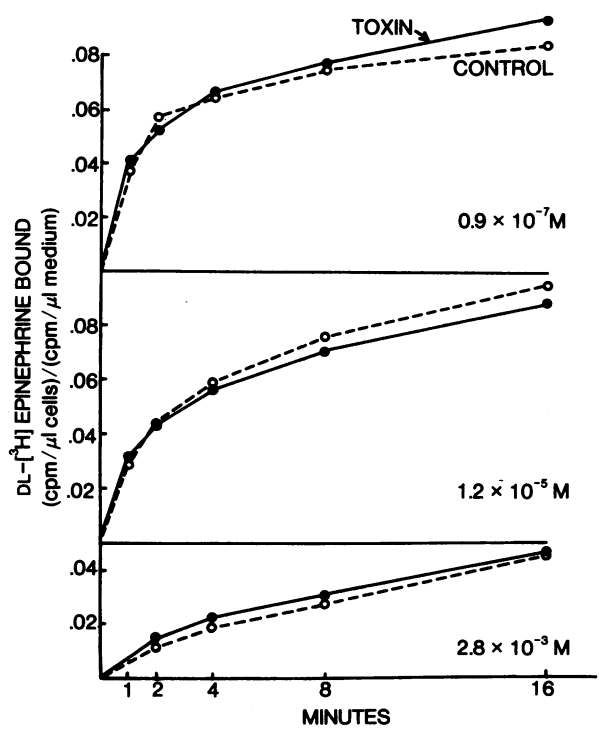

FIG. 5. Uptake of DL-[^{3}H]epinephrine by intact erythrocytes as a function of time and epinephrine concentration. Suspensions of toxin-treated (10 nM) and control erythrocytes were incubated in Tris–saline at 37° under oxygen. Cells were removed at various times after addition of DL-[^{3}H]epinephrine alone (0.9×10^{-7} M) or DL-[^{3}H]epinephrine and L-epinephrine (1.2×10^{-5} or 2.8×10^{-3} M). Results shown are for cpm of ^{3}H in Cl_3CCOOH supernates. Slopes of uptake become the same for all three epinephrine concentrations after 4 min. By subtraction of uptake data for 2.8×10^{-3} M epinephrine from data for the two lower concentrations, it can be shown that an equilibrium concentration-dependent binding is reached between 2 and 4 min. Each point is the mean of three experiments. Epinephrine concentrations are given at the *right*.

choleragenoid (10 nM). Increasing ATP concentration from 0.2 to 3.2 mM increased fluoride and epinephrine-augmented cyclase activities, but did not affect the relative differences between toxin-treated and control cells.

Epinephrine Uptake by Intact Erythrocytes. Epinephrine uptake, as determined from the amount of [^{3}H]epinephrine released from erythrocytes by Cl_3CCOOH, was the same for toxin-treated and control cells under a variety of conditions. Thus, at 37° no significant differences were observed for incubation times ranging from 1 to 16 min and for epinephrine concentrations in the range 10^{-7} M to 4.4×10^{-3} M (Figs. 5 and 6). Significant differences between toxin-treated and control cells were also not observed in 10 min incubations at 4° over the concentration range 2×10^{-8} M to 4.4×10^{-3} M (Fig. 6). Furthermore, there was no significant difference between toxin-treated and control cells with respect to cpm of ^{3}H released by the cells into the initial buffer wash solutions (data not shown). Finally, thin-layer chromatography of acid extracts from cells incubated at 37° for 10 min with 5 μM DL-[^{3}H]epinephrine revealed identical patterns for toxin-treated and control cells. More than 80% of the bound ^{3}H represented epinephrine.

DISCUSSION

Cholera toxin-treated erythrocytes showed an increase in maximal epinephrine-stimulatable adenylate cyclase activity and a decrease in the apparent K_m of the reaction for epinephrine. In principle, the toxin could produce these changes in three ways: (*i*) by altering epinephrine binding to receptors, (*ii*) by modifying the membrane constituents (presumably phospholipids) that couple catecholamine receptors to the catalytic component of the enzyme system, or (*iii*) by modifying the catalytic component itself, rendering it more susceptible to hormonal activation. On the basis of epinephrine binding studies, the first of these possibilities must be considered very unlikely. It would be hazardous at present to attempt to choose between the other two possibilities.

Since cholera toxin reduces the apparent K_m of the adenylate cyclase system for epinephrine without appearing to alter the kinetics of epinephrine binding to receptors, there cannot be a simple one-for-one relationship between receptors and enzyme molecules. Either there must be considerable cross-linking between receptors and enzyme molecules or the total number of receptors must greatly exceed the total number of enzyme molecules. Toxin could then increase the extent of cross-linking or modify existing bonds so as to reduce the number of communicating receptors that must be complexed with epinephrine before activation of a given enzyme molecule can occur.

The toxin-induced increase in maximal epinephrine-stimulatable cyclase activity appears to develop at the expense of maximal fluoride-stimulatable activity that is proportionately decreased by toxin (the sum of basal, maximum epinephrine-stimulated and maximum fluoride-stimulated activities is not affected). The distinction between epinephrine and fluoride-stimulatable cyclase activities is further emphasized by the nearly additive effect obtained when maximally stimulating amounts of both agents were added in combination. Additive effects from combined additions of fluoride and hormones have not been generally observed. Harwood and Rodbell (23) have suggested that this may be the case because fluoride usually has a dual effect, stimulating cyclase directly but also "uncoupling" hormone receptors

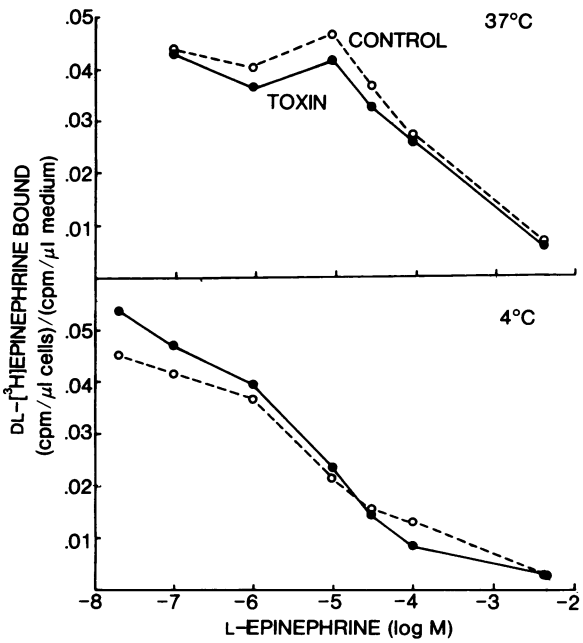

FIG. 6. Uptake of DL-[^{3}H]epinephrine by intact erythrocytes as a function of epinephrine concentration. Results shown are for cpm of ^{3}H in Cl_3CCOOH supernates and have not been corrected for nonspecific uptake (uptake at 4.4 mM). Incubations at 37° were for 1 min and incubations at 4° were for 10 min. Each point is the mean of four or five experiments. There were no significant differences between toxin-treated and control cells. Uptake is significantly greater at 37° than at 4° for epinephrine concentrations of 10 μM and above. The uptake of ^{3}H at 37° is significantly greater at 10 μM than at 1 μM epinephrine. The reason for this is not known.

from the catalytic component of the enzyme system. In the turkey erythrocyte this uncoupling effect would appear to be minimal.

The above observations suggest the possibility that the catalytic component of the turkey erythrocyte's adenylate cyclase system is capable of transformation from a catecholamine-sensitive, fluoride-refractory state to a fluoride-sensitive, catecholamine-refractory state. Levey (24) has shown that myocardial adenylate cyclase loses its hormone responsiveness when it is solubilized, but retains its fluoride sensitivity. Hormone responses can then be restored by adding certain phospholipids to the solubilized enzyme (25–27). Thus, loss of catecholamine sensitivity and enhancement of fluoride sensitivity may reflect a weaker association between the enzyme molecules and the phospholipid matrix of the plasma membrane. Cholera toxin appears to stabilize the enzyme in its catecholamine-sensitive (receptor-coupled) state.

Addendum

Two recent reports are relevant to the present findings. Enhanced adenylate cyclase activity in response to catecholamines has been demonstrated in toxin-treated liver membranes by Beckman *et al.* (28) and in toxin-treated fat cells by Hewlett *et al.* (29). Beckman *et al.* (28) also found that toxin-enhanced activity was retained by liver cyclase after solubilization with Lubrol-PX and that the solubilized enzyme from toxin-treated membranes (but not control membranes) retained some catecholamine sensitivity. Conceivably a membrane fragment was solubilized that contained both protein and phospholipid.

The author is indebted to Ms. Antonia Henderson and Mr. Phillip L. Smith for assistance with these experiments and to Dr. Richard A. Finkelstein for a gift of purified choleragenoid. This work was supported by Grants AI-09029 (U.S.-Japan Cooperative Medical Science Program) and AM-05114 from the National Institutes of Health.

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