Interaction of cholera toxin and *Escherichia coli* enterotoxin with isolated intestinal epithelial cells

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HYUN, CHUL S., AND GEORGE A. KIMMICH. *Interaction of cholera toxin and Escherichia coli enterotoxin with isolated intestinal epithelial cells.* Am. J. Physiol. 247 (Gastrointest. Liver Physiol. 10): G623–G631, 1984.—The interaction of biologically active ^{125}I-labeled cholera toxin with isolated chick intestinal epithelial cells involves a large number (approx 1.7 10^6/cell) of high-affinity ($K_d = 8–9 \times 10^{-9}$ M) binding sites that belong to a single class. Binding of iodotoxin to the cells occurs rapidly, is half-maximal within 1 min, and is complete in 3–7 min (at 37°C) depending on the toxin concentration. Toxin binding is saturable and includes only a small contribution from nonspecific sites. Ligand competition studies suggest that the isolated B subunit of choleragen (CT-B) behaves in an almost identical fashion to the holotoxin (CT), whereas the A subunit shows no detectable activity in competitive binding. Assays for cAMP indicate that neither that A nor the B subunits of CT contain any activity for increasing the level of intracellular cAMP. B subunit, when incubated with CT, inhibits CT-induced elevation of cAMP in a dose-dependent manner. Preincubation of ^{125}I-CT with various concentrations of ganglioside GM_1 also shows a dose-dependent inhibitory effect on the binding activity of the toxin. Pretreatment of CT with increasing concentrations of GM_1 results in a progressive decrease in toxin-induced formation of cAMP. *Escherichia coli* heat-labile enterotoxin, which is known to alter intestinal function via a mechanism similar to that of CT, has binding and biological effects very similar to those of CT.

radioligand binding; monosialoganglioside GM_1; membrane receptors; cAMP

CHOLERA IS A DISEASE caused by the local action of a bacterial exoenterotoxin on the small intestine and is characterized by a profuse intestinal secretion of fluid and electrolytes (2, 14). The bacterium responsible for the disease is *Vibrio cholerae*. Once established, the vibrio grows in the gut and secretes the exotoxin, which binds to receptors on the intestinal mucosa to cause subsequent physiological effects that are believed to be mediated by an elevation of cAMP levels in the mucosal epithelial cells (14, 15, 24).

The past decade has brought considerable progress in elucidating the structure of choleragen and its subunits (19, 29, 38) and in identifying the cellular membrane receptor (6, 8, 18, 39), as well as in understanding the mechanism by which the toxin activates adenylate cyclase in nontarget cells (3, 12, 21, 34). Choleragen is an oligomeric protein of 84,000 daltons and is composed of ated (28, 38). The intact toxin contains five monomers of subunit B, each of which possesses a binding site for attachment to the external surface of the target cell membrane (19). The A subunit is composed of two polypeptide chains, A_1 and A_2, linked by a disulfide bridge (19, 38). The A_1 peptide has enzymatic activity and catalyzes ADP-ribosylation of specific cellular proteins, which undergo a change in function and account for the action of the toxin (12, 21).

The action of choleragen as established by these studies with various experimental model systems can be divided into two separate events. The initial step is the interaction between the B subunit and specific cell membrane receptors in an orientation that presumably is favorable for the penetration of the A subunit through the plasma membrane into the cytosol (33). It has been demonstrated that ganglioside GM_1 is the native receptor for cholera toxin (CT) in the intestinal brush-border membrane as well as in other systems (6, 7, 31, 33). Binding interaction is believed to be followed by penetration of the A subunit, intracellular release of the A_1 peptide, and subsequent ADP-ribosylation of the N protein, which in turn modifies activity of the adenylate cyclase complex (3, 21). The ribosylation results in activation of adenylate cyclase due to inhibition of GTP hydrolysis by N protein and consequent retention of the cyclase in an active state (4).

Although much work has been accomplished, most of these findings come from work done on model systems not closely related to the pathophysiology of cholera so that comparatively little is known about the kinetic characteristics of CT binding to the physiological target cell. The following work describes studies aimed at contributing to our understanding of the interaction between CT or its subunits and intestinal epithelial cells, which represent the usual in vivo locus of toxin action. Because *Escherichia coli* heat-labile enterotoxin (LT), which is comprised of polypeptides of similar size to the A and B subunits of CT (5, 9, 20, 37), has been shown to alter intestinal transport function in a manner similar to CT (11, 13, 32), its interaction with enterocytes and its biological activity have been included and compared with those of CT.

MATERIALS AND METHODS

Cell isolation and preparation. Intestinal epithelial cells

tinal tissue of 6-wk-old White Leghorn chickens by use of the hyaluronidase-mechanical agitation method described by Kimmich (25). The isolated enterocytes were suspended in a mixed salts medium at a final concentration of 2–20 mg cell prot/ml. The composition of the suspension medium depended on the requirements of the particular experiment and is given in the appropriate figure legends. Experiments were performed in a medium containing 80 mM NaCl, 10 mM mannose, 55 mM Tris-HCl (pH 7.4), 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM glutamine, 0.5 mM β-hydroxybutyrate (Sigma Chemical), and 1 mg/ml bovine serum albumin (BSA, bovine testes, Sigma type I). Cell protein concentration was determined by the biuret procedure using BSA as the standard.

Isolation and purification of CT and its subunits. V. cholerae strain 569B was used for the production of CT and its subunits. The toxin was isolated and purified according to the procedure of Mekalanos et al. (30). Cells were inoculated to a final cell density of 5×10^6 cells/ml of Cye broth (30 g Casamino acids/l and 5 g yeast extract/l, adjusted to pH 7.8 with 5 N NaOH and autoclaved). A and B subunits of CT were isolated from pure CT by gel filtration (G-75 Sephadex column) under dissociating conditions according to the method of Klapper et al. (26). Purity of toxin and its subunits were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Toxin iodination. Radioiodinated CT (^{125}I-CT) was prepared by the chloramine-T method (8) with minor modifications. Briefly, a 5-mCi (10-μl) aliquot of $Na^{125}I$ solution (New England Nuclear) was added to 5 mg of pure choleragen in 1 ml of phosphate-buffered saline (PBS) (pH 7.4) and 40 μl of KI (1 mg/ml) in a glass beaker (3.5 x 4 cm) surrounded by ice. One milliliter of chloramine-T (1 mg) was then added dropwise to the beaker. The reaction mixture was stirred continuously with a small polyethylene-coated magnet, and the reaction was terminated by addition of 0.5 ml of sodium metabisulfite solution (50 mg) and 1 ml of KI (33 mg) in PBS. The reaction mixture was dialyzed in PBS (pH 7.4) overnight against TEAN buffer at 4°C. Separation of labeled protein from unreacted iodine was achieved by gel filtration on a 2.5 x 9.0 cm column of Sephadex G-25 equilibrated with TEAN buffer. (TEAN contains 0.2 M NaCl, 0.05 M Tris, 0.001 M EDTA, and 0.003 M NaN_3 and was adjusted to pH 7.4 with HCl.) After removal of free iodine, more than 99.5% of the radiolabeled material could be precipitated with 30% trichloroacetic acid (TCA). The percentage of total radioactivity incorporated into protein was always near 40%, and the specific activity of the radioiodinated toxin varied between 0.5 and 2.5 μCi/μg CT, depending on the concentration of CT and the specific activity of $Na^{125}I$ (carrier free) employed for iodination. The iodinated CT was stored at 4°C in TEAN and was used within 20 days. Under these conditions, ^{125}I-CT is stable for at least 30–40 days.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. SDS-PAGE was performed on ^{125}I-CT according to the procedure developed by Laemmeli (27). After electrophoresis system and by slicing and counting 2-mm sections. Relative mobilities of the iodinated subunit proteins were the same as those of the subunits of native CT. Biological activity of iodotoxin was evaluated by its activity in elevating cellular cAMP levels in comparison with native toxin. The protein concentration of CT, ^{125}I-CT, CT-A, and CT-B were determined by the method of Bradford (1) as well as by 280-nm absorption. The values of absorbance at 1%/cm (280 nm) for CT and CT-B were assumed to be 11.42 and 9.56 (29), respectively.

cAMP assay. The isolation and assay of cAMP in cell extracts were accomplished as described previously for this cell preparation (23). Briefly, after incubating the isolated enterocytes at 37°C for various intervals with or without test agent(s) in the medium, 200-μl aliquots of cells were removed from the reaction beakers and diluted in 2 ml of ice-cold medium. The chilled diluted sample was immediately centrifuged at 750 *g* for 30 s in order to pellet the cells. After resuspension in fresh ice-cold medium, the 30-s centrifugation step was repeated for thorough washing of the cells. The cell pellets were then extracted with 1 ml of 8% TCA. A subsequent centrifugation at the same speed for 10 min was employed to sediment coagulated protein. The cAMP contained in the supernatant was separated from TCA by chromatography using a 0.5 x 15 cm column of cation-exchange resin (Dowex 50X, hydrogen form, Sigma Chemical) equilibrated with 0.1 N HCl. cAMP was eluted from the column with deionized distilled water. The elution pattern for cAMP was monitored, and efficiency of recovery was calculated by addition of a trace amount of [^{3}H]-cAMP (New England Nuclear) to each cell extract prior to its application to the column. Average recovery of the radioactivity was greater than 98%. The effluents collected from these columns were assayed for cAMP content by radioimmunoassay employing the Schwarz-Mann assay kit.

Assay for binding of ^{125}I-CT to isolated enterocytes. The incubation conditions for this assay are similar to those of the cAMP measurements. Isolated enterocytes with protein concentrations of 0.2 mg–5 mg/ml were incubated at the indicated temperature with various concentrations of iodotoxin or with iodotoxin plus a 150-fold excess of unlabeled CT in a shaker bath. At desired intervals, 100-μl aliquots were removed, centrifuged at 750 g for 30 s, and washed three times with fresh cold media. The pellet in the final wash was counted in a well-type gamma counter (Packard Auto Gamma spectrometer model 3002) using disposable polypropylene tubes. Specific binding was determined as the difference between total binding (tubes containing ^{125}I-CT only) and nonspecific binding (tubes containing ^{125}I-CT plus a 150-fold excess of unlabeled CT). Unless otherwise specified, all values for binding of ^{125}I-CT to isolated enterocytes are corrected for nonspecific binding and expressed as specific binding. Scatchard analysis (36) was employed to determine the dissociation constant (K_d) and total number of binding sites (n).

Other methods and materials. Trypsin activation of *E. coli* LT was achieved by adding trypsin (Sigma Chemical)

was terminated by adding soybean trypsin inhibitor (Sigma Chemical) at the same concentration. Such trypsin treatment has been shown to nick the A subunit of LT (5). Unlike CT, in which subunit A is nicked during the process of isolation, LT-A must be experimentally activated by proteolysis. *E. coli* LT was prepared by Dr. John Clements in the Gastroenterology Unit at the University of Rochester Medical Center and generously supplied by his laboratory. Ganglioside GM_1 was purchased from Supelco (Bellafonte, PA).

RESULTS

As demonstrated in our previous report (23) freshly isolated chick enterocytes contain approximately 15 pmol cAMP/mg cellular prot and undergo a slow decrease in cAMP level during incubation at 37°C (Fig. 1). Incubation of enterocytes with CT induces a three-phase response consisting of a 10- to 15-min lag interval followed by a 40-min period of increasing cAMP content and an eventual steady state (Figs. 1 and 2), during which cAMP levels are approximately 10-fold higher than control levels. Also as shown in Fig. 1, when the cells are incubated with purified A or B subunit, no detectable elevation of cAMP level over the control cells is observed. However, there is an inhibitory effect of subunit B on the ability of CT to elevate cAMP concentration when the cells are incubated with both CT (0.5 μg/ml) and CT-B. The severity of inhibition is dependent on the concentration of B subunit and is almost complete at a CT-B concentration of 50 μg/ml. Subunit A does not cause any reduction in CT-induced cAMP elevation at the same concentration at which the suppression by B subunit is more than 50% complete (20 μg/ml).

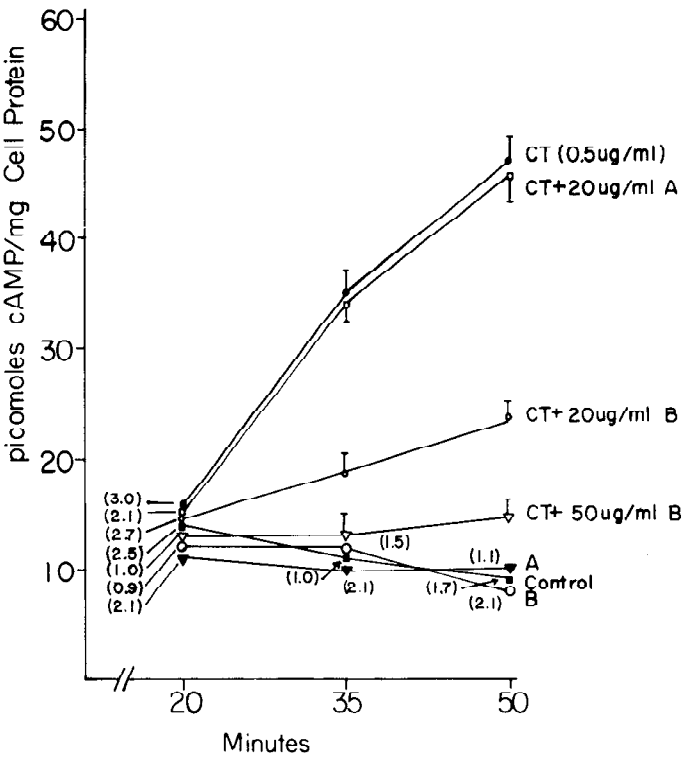

FIG. 1. Effects of CT-A and CT-B on cAMP levels of both control and toxin (CT)-treated cells. Concentrations: 0.5 μg/ml CT, 20 μg/ml CT-A, and 20 μg/ml CT-B, unless indicated otherwise. CT and subunits were added at same time (*time 0*). Each *point* is mean of 3 separate

Trypsin-activated *E. coli* heat-labile enterotoxin (3 μg/ml) causes an increase in intracellular cAMP in a manner similar to that observed for CT (Fig. 2). Trypsin has no effect on the activity of CT, but in the absence of trypsin activation a much longer lag phase is apparent for LT. Chlorpromazine inhibits the effect of either LT or trypsin-activated LT on cellular cAMP levels as we have also reported for CT (23). When the cells are incubated at 30°C for 50 min in the presence of various concentrations of trypsin-treated LT, a dose-dependent response is obtained that is maximal at about 6 μg/ml (90 pmol cAMP/mg cell prot). Half-maximal response is elicited at approximately 0.5 μg/ml (0.2×10^{-9} M) of trypsin-treated LT. Furthermore, the ability of trypsin-treated LT to elevated cAMP content is inhibited by CT-B (Table 1) in a manner similar to the inhibitory effect of CT-B on the activity of CT (Fig. 1).

When iodinated toxin was employed in order to monitor toxin binding to the isolated cells, the data presented in Fig. 3 were obtained. Note that the binding of ^{125}I-CT to enterocytes at 37°C occurs rapidly and is half maximal within 1 min. As the concentration of iodotoxin is increased, the amount of bound iodotoxin increases but the time required to reach the steady state decreases slightly. For instance, when the cells are incubated with 2.8 μg/ml ^{125}I-CT at 37°C, binding is complete in 3–5 min and the steady state is maintained during a subsequent 50-min incubation period. At 0.045 μg/ml about 10 min are required to reach the steady state. The rapidity of binding is in sharp contrast with the 15-min lag time observed for the CT-induced elevation of cellular cAMP level (Fig. 1). When binding is compared at different temperatures, it can be shown that a decrease in incubation temperature from 37° to 15°C causes a reduction in the rate of the binding reaction without a significant change in the maximum amount of iodotoxin that can be bound (Fig. 3).

The amount and stability of CT binding suggest the presence of a large number of toxin binding sites and a relatively slow turnover of the receptor sites. Toxin binding is also readily reversible at 37°C. If the cells are preexposed to ^{125}I-CT until binding equilibrium is attained, almost all (>95%) of the bound toxin is released within 70 min when excess unlabeled toxin was added subsequently (Fig. 4). These data indicate that only a minor portion of the bound toxin is internalized by the cells during the incubation interval.

When the specific binding of iodotoxin as a function of cell concentration is studied, it can be shown that at high cell densities only 70% of the iodinated toxin will bind (data not shown). If the cells are removed and the remaining 30% of iodotoxin in the supernatant is tested for binding to freshly isolated enterocytes, no detectable binding can be demonstrated. This lack of binding activity may be due to protein denaturation during isolation and iodination of the toxin and/or to changes in toxin

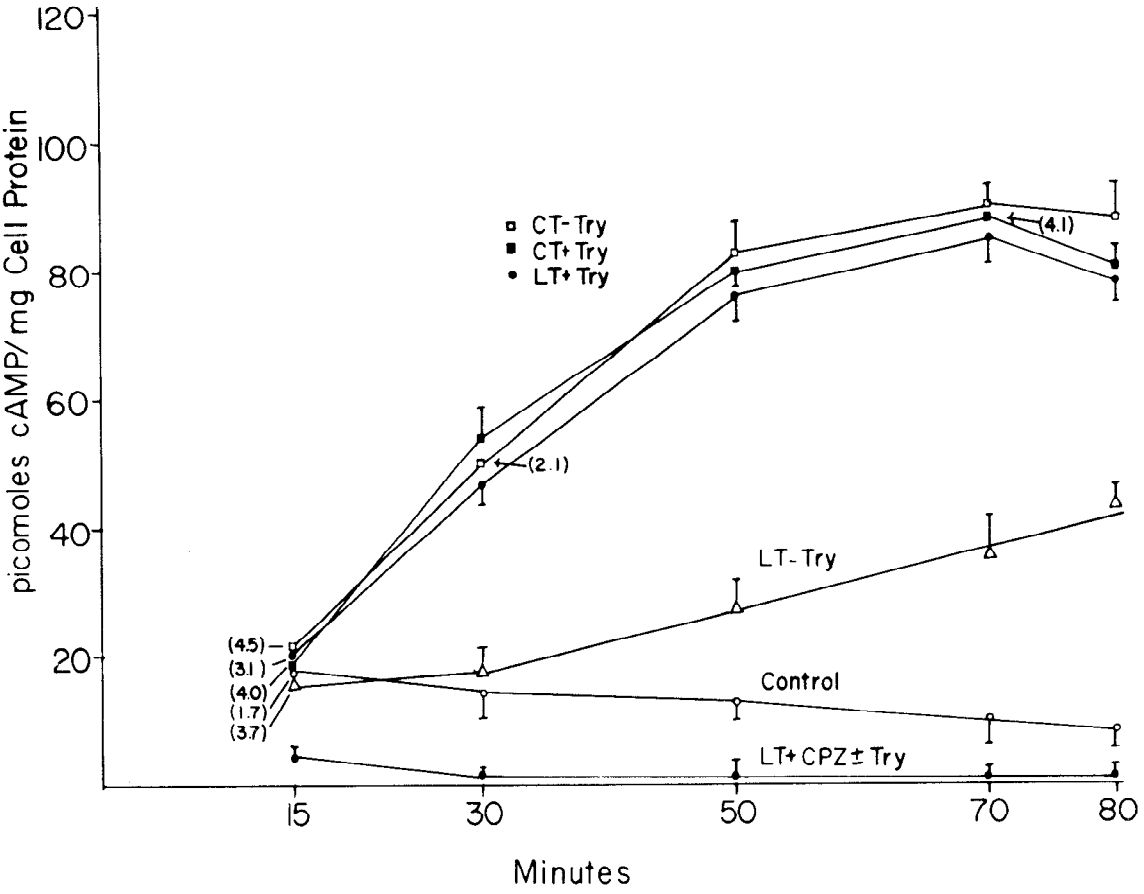

FIG. 2. Time-independent effects of cholera toxin (CT, 3.0 μg/ml) and *Escherichia coli* LT (3 μg/ml) on elevation of cAMP levels in isolated chick intestinal epithelial cells. Chlorpromazine (CPZ, 0.05 mM) and heat-labile toxin (LT) were added at same time (*time 0*). (See EXPERIMENTAL PROCEDURES for details of trypsin treatment). Each *point* represents mean ± SE for 3 separate determinations. Numbers in parentheses are SEs for indicated points.

TABLE 1. *Effect of subunit B on cAMP levels of enterocytes treated with trypsin-activated Escherichia coli heat-labile toxin*

Trypsin-Activated LT, μg/ml	CT-B, μg/ml	cAMP, pmol/mg cell prot
0	0	9.0 ± 2.9
0.5	0	45.1 ± 4.7
0.5	10	30.6 ± 3.1
0.5	20	25.5 ± 3.2
0.5	50	8.1 ± 4.0

Values are means ± SE for 3 separate determinations from 3 different cell preparations. Aliquots of an enterocyte suspension were incubated for 50 min with 0.5 μg/ml of trypsin-treated heat-labile toxin (LT) with different amounts of cholera toxin subunit B (CT-B) prior to assay for cAMP. The first line shows the result with no added agents. When added, LT and CT-B were added at the same time at the start of the incubation.

Figure 5*A* shows binding data for two cell populations differing in cell density in which each population was incubated with various concentrations of ^{125}I-CT or ^{125}I-CT plus a 150-fold excess of native CT. Specific and nonspecific binding is shown for the concentrated cell preparation (200 μg cell protein/ml), whereas only specific binding is shown for the dilute cell preparation (30

toxin binding. Figure 5*B* is a Scatchard plot of the same data using values for specific toxin binding. The equilibrium dissociation constants (K_d) for the concentrated and dilute cell populations were 9.2 and 8.4 nM, respectively. The value n (2.8 pmol CT/mg cell prot) represents the total amount of CT bound to 1 mg of cell protein, which represents approximately 10^6 cells in the enterocyte preparation. In both cases, approximately 1.7×10^6 iodotoxin molecules per cell were bound at equilibrium. (This number is obtained by multiplying the value of n by Avogadro's number and dividing it by 10^6 cells, i.e., 28×10^{-12} mol CT/10^6 cells $\times$ 6.023×10^{23} molecules/mol $\cong$ 1.7×10^6 molecules CT/cell.) The linearity of the Scatchard plot suggests no detectable site cooperativity and is indicative of a single class of binding sites.

Figure 6 shows that the specific binding of ^{125}I-CT to enterocytes is competitively inhibited by native CT, *E. coli* LT, and CT-B but not by CT-A. The pattern of inhibition of iodotoxin binding by these competitive agonists is quite similar. The degree of competition suggests CT-B and native CT have nearly equal affinity for the receptor site, whereas LT has a slightly lower affinity. When a saturating concentration of iodotoxin (2.98×10^{-7} M) was employed to study the competition by native toxin (Fig. 6, inset), half of the binding was inhibited by

the dissociation constants for the iodotoxin and native toxin binding are the same.

Ganglioside GM_1 inhibits binding of iodotoxin to enterocytes in a dose-dependent manner (Table 2). When iodotoxin (0.18 μg/ml) was incubated at 37°C with varying concentrations of GM_1 prior to incubation with the cells (1.5 mg cell protein/ml), half-maximal inhibition was achieved by 10 ng/ml (6.45 nM) of GM_1, and the binding was almost completely blocked by 125 ng/ml (8.06×10^{-8} M). Parallel experiments that monitored the amount of toxin-induced cAMP generated showed a good correlation between toxin binding and cAMP production (Table 2). When the same experiments were performed with trypsin-treated LT (0.18 μg/ml), a similar pattern of inhibition of toxin (LT)-induced elevation of cAMP by GM_1 could be demonstrated (data not shown).

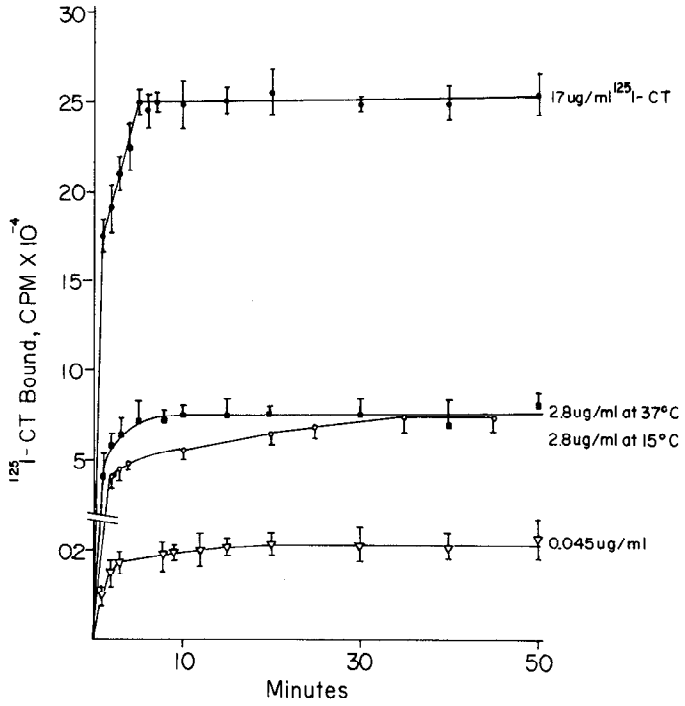

FIG. 3. Time course and temperature dependence of toxin binding. Enterocytes (1.12 mg cell prot/ml) were incubated at 37°C with different concentrations of ^{125}I-cholera toxin (sp act 1 μCi/μg). Concentrations: 0.045, 2.8, and 17.0 μg/ml. Specific binding was determined by method described in EXPERIMENTAL PROCEDURES. Values are means ± SE for 3 separate experiments.

DISCUSSION

We have recently described the fundamental characteristics of the action of cholera toxin in elevating intracellular cAMP levels in isolated enterocytes and related changes in membrane permeability to sodium (23). The present study focuses on the binding of toxin with receptors on the cell membrane, which represents the primary event in initiating the cellular action of toxin.

The results of these studies clearly demonstrate that toxin binding to intestinal epithelial cells is reversible, rapid, saturable, highly specific, and temperature dependent. The K_d values for the concentrated and diluted cell populations were 9.2 and 8.4 nM, respectively, when each data set was fit to a single Michaelis-Menten function, $B = T[F]/K_d + [F]$ (Fig. 5*B*). If a two-site binding model was employed

$$\left(B = \frac{T_1[F]}{K_{d1} + [F]} + \frac{T_2[F]}{K_{d2} + [F]}\right)$$

for the higher-density cell population (where B is bound toxin, F is free toxin, and T_n is total bound at saturation to site n), then fitting by an iterative process with the aid of a computer provided a K_d of 8.7 nM for the dominant site and a maximum binding of 5.1 pmol/mg prot to this site. The best fit occurred for a second-site K_d of 0.235 mM and maximum binding of 1,000 pmol/

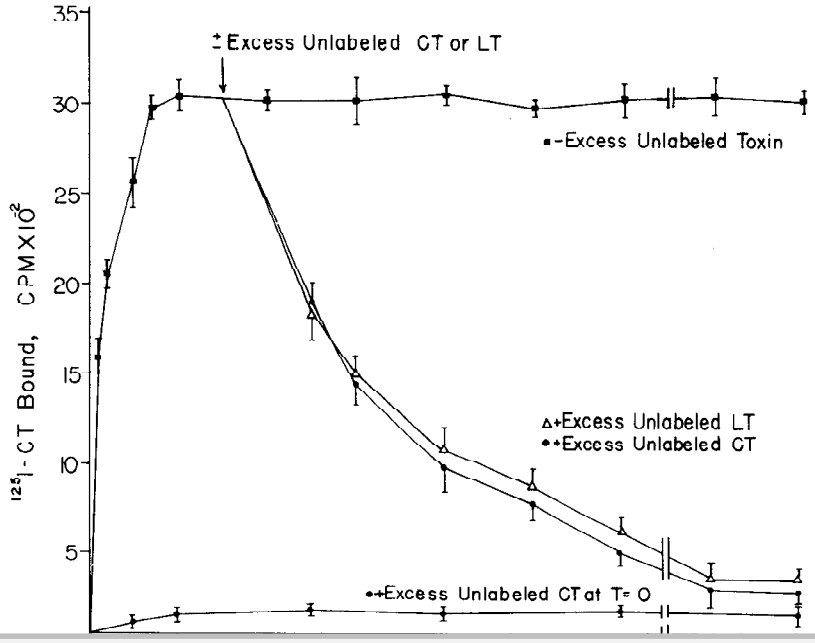

FIG. 4. Reversibility of ^{125}I-cholera toxin (CT) binding. Isolated enterocytes were incubated at 37°C with ^{125}I-CT in presence or absence of excess unlabeled CT to determine specific binding. After 15-min exposure to ^{125}I-CT, cells were incubated at 37°C with or without 150-fold concentrations of unlabeled CT or heat-labile toxin (LT) for following 75 min. Cell aliquots were then removed at indicated periods and assayed for total toxin binding. Each *point* is mean ± SE for 3 separate determinations.

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