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Heart and T-Lymphocyte Cell Surfaces Both Exhibit Positive Cooperativity in Binding a Membrane-Lytic Toxin

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Abstract. Cobra venom cytotoxins (CTX) have been shown to disrupt cells as different as immunocytes, skeletal myocytes, erythrocytes and tumor cells. Nevertheless, even subpopulations of tumor cells are differentially susceptible to CTX by an order of magnitude. In the present study, our objective was to compare CTX-specific binding with cytolytic potency for two disparate cell types in vitro. We investigated the lytic activity of cytotoxin-III from *Naja naja atra* (NNA, fraction D) using heart cells and human leukemic T-cells (CEM cells). For both cell types, 50% cytolysis, assessed by tetrazolium dye conversion, occurred with μM concentrations of toxin (EC_{50} = 2.2 μM). We examined the binding of radiolabeled CTX III to both heart cells and CEM cells and found the apparent dissociation constant ($K_{D_{app}}$) to be 0.69 μM and 0.75 μM, for CEM and heart cells respectively. The B_{max} for the CEM cells was 1.0 fmoles/cell and that for heart cells was 5.2 fmoles/cell, both exhibiting positive cooperativity between the sites (Hill coefficients 1.4, T-cells; 1.6, heart). Relatively modest dissociation constants plus high numbers of binding sites per cell are consistent with a model of CTX binding to plasma membranes by interaction with phospholipids in the bilayer. Our results suggest that the lytic activity of this cytotoxin follows its binding to a population of sites on the cells in a cooperative fashion.

Key words: Cardiotoxin — Cooperative Binding — Cytolysis — Cytotoxin — Leukemia

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Introduction

Cobra venom cytotoxins (CTX) are polycationic yet hydrophobic proteins, 60 amino acids in length [12]. Structurally, they contain primarily β-sheet and are rather planar molecules consisting of three loops protruding from a globular region in which are located four disulfide bonds [2]. There are more than 50 CTX variants which have been purified from different cobra venoms. Their functions are characterized by a variety of biological activities which include cardiac contraction [20], irreversible depolarization of excitable cells [20], tumor cell cytolysis [7], and lysis of erythrocytes [8]. How they cause cellular destruction has not yet been established, although a variety of mechanisms have been suggested over the years [18, 12, 19]. Considering the differences in lytic concentrations and the breadth of susceptibility among different kinds of cells, Menez et al. [31] suggested that individual CTX variants may exhibit unique modes of action upon various cells.

Our interest in these toxins evolved from their selectivity for T-lymphocytes over B-cells and macrophages [22]. We have shown that murine tumor T-cells are two-to-four times more susceptible to CTX-mediated cytolysis than are murine tumor B-cells [21]. Similarly, human leukemic T-cells are at least twice as susceptible to CTX as human leukemic B-cells (CEM vs. Daudi cell lines, *unpublished observations*). Management of immune system disorders involving certain populations of T-lymphocytes, for example, could benefit from the application of cell-selective agents, particularly in view of the ability of CTX to disrupt the membrane of a target cell without having to be internalized [21, 23]. However, the apparent recognition by native CTX of a number of different targets, particularly cardiac cells, potentially limits their therapeutic utility. Nevertheless, other toxins, such as pseudomonas exotoxin, have been

demonstrated to possess a cell recognition site which is distinct from its lytic domain [13]. Before one could determine whether the cytotoxins similarly possess distinct binding and lytic domains, information about their binding and lytic parameters needs to be obtained.

The present study investigates the lytic as well as the binding activity of one CTX variant (fraction D, or cardiotoxin III) isolated from *Naja naja atra* venom (CTX III). This CTX variant demonstrates cytolytic activity towards both heart cells and a leukemic T-cell line. Different laboratories have reported conflicting views with regard to the reversibility of toxin binding to cell surfaces [36, 4]. We therefore examined aspects of CTX binding which included saturability, cooperativity, reversibility, inhibition by nonradiolabeled CTX, and the kinetics of association and dissociation. To our knowledge, this is the first time such a comparison of cytotoxin lytic and binding parameters has been reported for distinct types of cells.

Materials and Methods

Materials

Cobra venom was purchased from the Miami serpentarium (Punta Gorda, FL). BioRex 70 was purchased from BioRad laboratories (Richmond, CA). Cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD). We chose a rat cardiac myocyte line rather than a primary embryonic explant as a source of heart cells in order to enhance homogeneity among the cells whose binding we wanted to investigate. Ross Park Memorial Institute Medium (RPMI) 1640, Hanks Balanced Salt Solution and Trypsin-EDTA were from Gibco BRL (Grand Island, NY); Penicillin/Streptomycin, L-Glutamine, Dulbecco's Modified Eagle's Medium, phosphate buffered saline and MTT dye were from Sigma (St. Louis, MO); all cell culture plates, pipettes and flasks were purchased from Fisher Scientific (Itasca, IL). [^{3}H]-Thymidine and Na^{125}I were from ICN Radiochemicals (Irvine, CA). Liquiscint aqueous scintillation solution was purchased from National Diagnostics (Manville, NJ); Scintillation vials were from Kew Scientific (Columbus, OH). Iodobeads were from Pierce (Rockford, IL); Polystyrene conical tubes were from Sarstedt (Newton, NC); 96 well removable polystyrene plates were purchased from Dynatech Laboratories (Chantilly, VA). All other chemicals were from Sigma, and all other glassware was from Fisher Scientific.

Toxin Purification

Naja naja atra venom toxins were purified by the method of Karlsson et al. [24], and cytotoxin-III (CTX) was obtained following the procedure outlined in Zusman et al. [40], which yields a product devoid of phospholipase A_2 activity. Protein concentrations were determined by the method of Lowry et al. [29]. Purity of each fraction was assessed by high pressure liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and amino acid composition determination.

Cell Culture

Human leukemic T-lymphocytes (CEM cells) were cultured in RPMI 1640 medium supplemented with 20% fetal bovine serum, 50 U/ml penicillin and 50 mg/ml streptomycin. Cells that were at least 95% viable (Trypan blue exclusion) were used in all experiments before reaching a maximal concentration of 2×10^6 cells/ml. Heart cells (line: H9C2) were cultured in glucose-supplemented Dulbecco's Modified Eagle's Medium containing 15% fetal bovine serum, 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine. For each experiment, heart cells at 90% confluence were removed from growth flasks using 0.05% trypsin containing 0.53 mM EDTA. Both cell types were maintained at 37°C in a humidified incubator containing 5% CO_2.

Toxicity Assays

To determine the cytotoxicity of CTX III for the cells, two methods of determining viability were used. In both cases, wells containing only cells without toxin were used to indicate maximum control viability, and all values of percent were determined as a fraction of the mean control values for each experimental condition.

Vital Dye Incorporation

The ability to oxidize MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was used as a measure of cell viability. The procedure was modified from Hansen et al. [17] as follows: cells in fresh supplemented RPMI 1640 (2×10^5 CEM cells or 1×10^5 Heart cells) were added to 96-well flat bottom plates. CTX III at varying concentrations was then added to duplicate wells, and the plates were incubated at 37°C, 5% CO_2 for 1 hr. To each well was then added MTT in phosphate-buffered saline (1 mg/ml final concentration), and these were subsequently incubated for 2 hr at 37°C. Following this incubation period, 100 μl of lysing buffer (20% sodium dodecyl sulfate in 50% dimethyl sulfoxide) was added to the wells, which were then incubated overnight at 37°C. The final volume in the wells was 225 μl. For each well in the experiment, absorbance at 570 nm was measured using wells with medium and dye plus lysing buffer as the zero reference controls. Although all cells incorporated the dye, only viable cells were able to oxidize it, causing a change in color from yellow to blue.

Thymidine Incorporation

To duplicate wells containing 2×10^5 cells was added 100 μl of CTX III at varying concentrations, and the plates were incubated for 1 hr at 37°C and 5% CO_2. [^{3}H]-thymidine (1 μCi) in 25 μl of medium was added to each well, and the plates were incubated overnight at 37°C. The next day, cells were harvested onto glass fiber filters. The filters were allowed to dry, after which they were placed in 5 ml of scintillation fluid and counted in a beta counter.

Binding Experiments

General Procedure

Iodination of CTX III was achieved following the procedure of Markwell et al. [30]. Following iodination, the toxin was dialyzed against deionized water, lyophilized and stored at 4°C in 1 mg fractions. All binding assays were performed in an assay buffer: 50 mM Tris-HCl, pH 7.2, with 0.1% bovine serum albumin (BSA). Wash buffer consisted of 50 mM Tris-HCl, pH 7.2. Assays were performed in removable 96-well plates coated with 0.1% BSA prior to use. All toxin dilutions were done in 13 × 100 mm borosilicate glass tubes which had been previ-

ously coated with 0.1% BSA. Coating was essential to minimize adherence of the toxin to solid surfaces. For all experiments, each sample was prepared in triplicate.

We were interested in determining if external cell surface binding sites for the CTX were the same for T-lymphocytes and heart cells. For this reason, whole cell preparations rather than cell membranes were used. Charge-dependent, nonspecific binding to intracellular membrane proteins available in cellular lysates or in microsomal preparations would have rendered investigation of specific external binding sites unfeasible.

Determination of nonspecific binding was complicated for two reasons: First, CTX has been found to nonspecifically adsorb to surfaces such as plastic or glass; second, the high concentrations of unlabeled toxin required for conventional determination of nonspecific binding would potentially lyse cells and release intracellular proteins to which toxin could subsequently bind. Concerning the first challenge, our efforts to reduce nonspecific binding eventually led us to apply BSA as a coating agent for the adsorptive surfaces. Despite the use of BSA to optimally reduce nonspecific binding, approximately 5% of the total bound CTX was still found to adsorb to the surface of the polystyrene wells. In the presence of unlabeled CTX, the extent of this nonspecific binding was significantly reduced. With regard to the second challenge, unlabeled ligand is normally used to saturate the specific sites of a receptor in the control wells of binding experiments, so that labeled ligand can only bind residual nonspecific sites in cell membranes. In the case of highly adsorptive peptides such as CTX, however, unlabeled ligand can bind not only specific sites, but also nonspecific sites: both cellular constituents and solid surfaces. Nevertheless, nonspecific binding measured in the presence of a 100-fold excess unlabeled toxin resulted in the same curvilinearity, the same K_Ds, and the same Hill coefficients as obtained without cells (*data not shown*). Since our goal was to compare and contrast the binding of the toxin to surface sites for both heart cells and T-lymphocytes, we chose to measure nonspecific binding as the amount of radiolabeled toxin that could not be washed off BSA coated wells. Our procedure of measuring nonspecific binding by using wells containing only labeled CTX therefore allowed for more conservative estimates of specific binding than using an excess of unlabeled CTX.

Saturation Binding

Varying concentrations (from 0.01 μM to 12 μM) of [^{125}I]CTX III were prepared, and 150 μl aliquots of each dilution were distributed into wells. CEM cells which had been washed twice with Hanks Balanced Salt Solution were resuspended in assay buffer at a concentration of 3.3×10^5 cells/ml. Heart cells which had been trypsinized as described above and washed once with phosphate-buffered saline were resuspended in assay buffer at a concentration of 6.7×10^4 cells/ml. To the wells containing [^{125}I]CTX III was added 150 μl of the cells, and these were then gently mixed and incubated for 1 hr at 22°C. Plates were then centrifuged at $500 \times g$ for 10 min, and unbound CTX was removed by vacuum filtration. The wells were washed twice with 300 μl wash buffer, gently vortexed and again centrifuged. Individual wells were placed in 13 × 100 mm test tubes and counted in a gamma counter. The values obtained were used as a measure of total binding. Nonspecific binding was determined by adding 150 μl of assay buffer to wells containing [^{125}I]CTX III at each concentration. These wells were incubated and washed similarly to the wells containing CEM or heart cells and also counted in the gamma counter. The specifically bound CTX was determined as:

$$\text{cpm}_{\text{specific}} = \text{cpm}_{\text{total}} - \text{cpm}_{\text{nonspecific}} \quad (1)$$

Data were then subjected to Scatchard and Hill analyses, and the K_D and number of binding sites/cell were determined as described by Bylund [6].

Association

A 1.0 μM solution of [^{125}I]CTX III was prepared in assay buffer, and 150 μl was transferred into BSA-coated microtiter wells. Previously washed cells in assay buffer (150 μl) were then added to the wells, and these were incubated at 22°C for various times. Bound toxin was again separated from free toxin by aspiration after the plates had been washed twice by centrifugation. The wells were counted in a gamma counter, and the results expressed as Specific Bound *vs.* Time (min). The value of k_{ob} (observed association constant) was determined as the slope of the line obtained from a plot of:

$$ln(B_e/[B_e - B]) = k_{ob}t, \quad (2)$$

where B_e = specific binding at equilibrium, and B = specific binding at any time t [6].

Dissociation

A 75 μl aliquot of previously washed cells in assay buffer containing 5×10^4 CEM cells or 1×10^4 heart cells was added to BSA-coated wells, and 75 μl of [^{125}I]CTX III (final concentration = 0.5 μM) was then added to the wells. These were allowed to incubate for 1 hr at 22°C. To the wells was then added 150 μl of a 100 μM solution of unlabeled CTX III (final concentration = 50 μM), and the wells were incubated at 22°C for varying times. Bound toxin was separated from the free toxin as just described. The values obtained from 15-min samples were used as the $t = 0$ point, since this was the minimum time that could be achieved experimentally. The data obtained were used to derive a plot of B/B_0 *vs.* Time (min), and the k_{-1} (dissociation rate constant) was determined from this plot as follows:

$$k_{-1} = -ln \text{ of } 0.5/\text{time at which } B = 0.5\ B_0, \quad (3)$$

where B_0 is the amount specifically bound at time $t = 0$ [6]. Using the values of k_{ob} obtained from the association plot and the k_{-1} obtained from the dissociation plot, k_{+1} (the association rate constant) was determined as follows:

$$k_{+1} = [k_{ob} - k_{-1}]/L_T, \quad (4)$$

where L_T = labeled ligand concentration used.

The K_D (equilibrium dissociation constant) was then calculated from the values of k_{-1} and k_{+1} obtained, using the formula:

$$K_D = k_{-1}/k_{+1} \quad (5)$$

Inhibition Experiments

[^{125}I]CTX III (75 μl) was added to each well to achieve a final concentration of 0.45 μM. To this was added 75 μL of unlabeled CTX III at various concentrations. Previously washed cells (5×10^4 CEM cells or 1×10^4 heart cells) in a volume of 150 μl were aliquoted into the wells, and these were incubated at 22°C for 1 hr. Bound toxin was separated from free toxin as described previously. Nonspecific binding was determined by using wells containing both labeled and unlabeled CTX at each concentration, without cells. The data were then used to generate a plot of percent Inhibitor Bound *vs.* Log Inhibitor concentration, where percent inhibitor bound was determined [6] as:

$$(B_y/B_T) \times 100\%, \quad (6)$$

where B_y = concentration of the bound inhibitor and B_T = total specific bound.

IC_{50} values were determined graphically, as the concentration required to obtain 50% inhibition of binding.

ABBREVIATIONS

BSA	bovine serum albumin
CTX	cytotoxin(s)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NNA-D	*Naja naja atra* cytotoxin, fraction D
RPMI	Ross Park Memorial Institute Medium

Results

As indicated by high pressure liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1), and amino acid composition determinations, CTX III from *Naja naja atra* was deemed to be greater than 90% pure. Our preparation of the CTX III was apparently devoid of phospholipase contamination, as can be seen in Fig. 1 and has been the case for other CTX samples purified using the method of Zusman et al. [40].

CTX III was shown to be toxic to both heart cells and human T-lymphocytes with activity in the μM range. From Fig. 2, cytolytic activity was concentration-dependent for both cells. Dose-response curves were identical for both cell types, with maximal toxicity observed to be nearly 90% in either assay. The concentration of CTX required to obtain 50% cytolysis (LD_{50}) was approximately 2 μM (Fig. 2*a*), and that required to prevent 50% cell division, as measured by inhibition of the incorporation of [^{3}H]-Thymidine, was 0.5 μM (Fig. 2*b*).

To ascertain the relationship between the lytic function of cytotoxins and their binding to cell surfaces, binding parameters of CTX III were examined using ^{125}I-labeled toxin. Specific binding of the toxin to either cell type was found to be saturable (Figs. 3*a* and 4*a*) and reversible (Figs. 5*b* and 6*b*). The values of K_Ds determined from saturation experiments were 0.69 ± 0.04 μM and 0.75 ± 0.02 μM for CEM and heart cells respectively (Fig. 3*a* inset and 4*a* inset). The CEM cells exhibited 1 fmole of binding sites per cell, while the heart cells were shown to have 5 fmole of binding sites per cell (Table). Scatchard analysis of the saturation binding data resulted in upwardly convex curves, indicating positive cooperativity between sites on either cell type (Figs. 3*b* and 4*b*). Values of Hill coefficients were determined to be 1.4 for the CEM cells and 1.6 for the heart cells (Fig. 3*a* and 4*a* insets), further supporting the positively cooperative nature of the binding of CTX III.

The results of the association and dissociation experiments are given in Figs. 5 and 6. Saturation of binding for both cell types was achieved at approximately 1 hr. Using the kinetic parameters derived from Figs. 5 and 6, the same value of K_D for the CEM cells was determined (0.76 μM). The value of K_D for the heart cells could not be determined because the dissociation curve (Fig. 6*b*) was a curvilinear plot which was non-monotonic. Inhibition binding experiments shown in Fig. 7 confirmed the saturation binding K_D values (0.65 μM for CEM cells and 0.79 μM for heart cells).

The Table summarizes the parameters, binding and lytic, of the CTX III cytotoxin for both CEM and heart cells. As can be seen from this table, this cytotoxin variant behaves similarly towards these two cell types, with the exception being the number of binding sites which each cell possesses.

Discussion

The above experiments indicate that similar cytotoxin-binding parameters were obtained using either human T-lymphocytes or rat heart myoblasts. For both types of cells, binding is saturable, reversible, and can be inhibited by an excess of unlabeled ligand. This agrees with the report of Borkow et al. [4] concerning toxin γ from *Naja nigricollis* binding to leukemic cells, but differs from the findings of Takechi et al. [36], who indicated that CTX binding to fetal lung cells was irreversible.

The ability of CTX to inhibit thymidine incorporation at concentrations significantly lower than those required to permeabilize the plasma membrane suggests that cell division may be inhibited before extensive membrane disruption occurs. This could reflect a protective signaling mechanism by the plasma membrane to the cell nucleus, as membrane integrity begins to be perturbed. On the other hand, this phenomenon could result from intrinsic structural cellular defense capabilities, whereby less severe perturbations to the cell membrane are accommodated without functional loss of membrane integrity [32, 34].

To measure binding of a cytolytic toxin to the surface of intact cells could be difficult for at least two reasons: (1) cellular disruption may allow toxin access to the interior of the cell; (2) toxin-induced cell lysis could permit intracellular proteins to diffuse out of the cell where they could subsequently bind the toxin. A comparison of Fig. 2*a* with Fig. 3*b* suggests that a sudden increase in CTX binding does not occur upon T-cell cytolysis: binding has saturated in the presence of 2 μM CTX, while the extent of cytolysis at that concentration, measured by inability of the T-cells to metabolize tetrazolium dye, is less than 50%; this conclusion is supported by the differences between the K_D and the LD_{50}. With regard to the second possibility, even if intracellular protein had escaped, it would have been lost during the wash procedures and would not have been measurable.

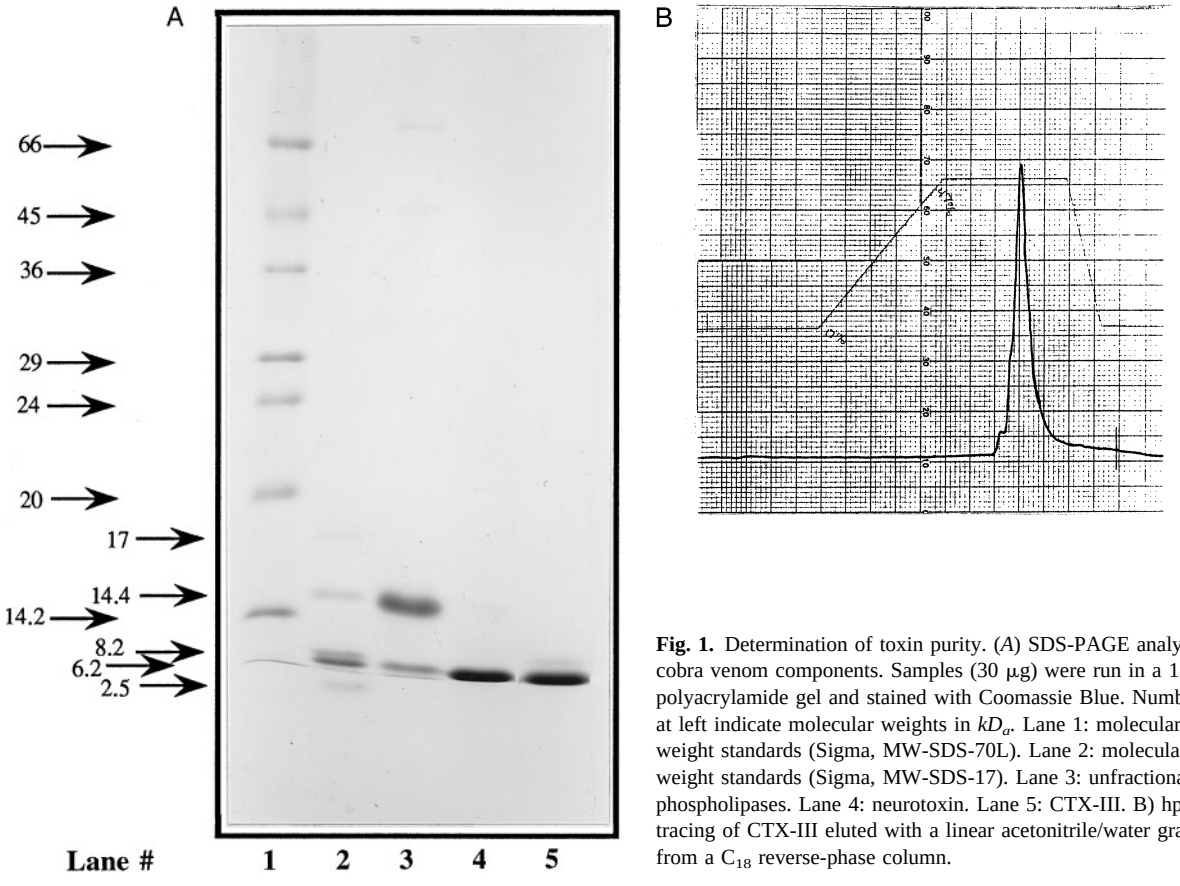

Fig. 1. Determination of toxin purity. (*A*) SDS-PAGE analysis of cobra venom components. Samples (30 μg) were run in a 15% polyacrylamide gel and stained with Coomassie Blue. Numbers at left indicate molecular weights in kD_a. Lane 1: molecular weight standards (Sigma, MW-SDS-70L). Lane 2: molecular weight standards (Sigma, MW-SDS-17). Lane 3: unfractionated phospholipases. Lane 4: neurotoxin. Lane 5: CTX-III. B) hplc tracing of CTX-III eluted with a linear acetonitrile/water gradient from a C_{18} reverse-phase column.

Positive cooperativity in cytotoxin binding to either cell type, as evidenced by convex Scatchard plots [10], was unexpected. In the only previous study in which a Scatchard plot of CTX binding data was provided, Takechi et al. [36] had reported a biphasic CTX III binding curve, with negative cooperativity. Our observation of positive cooperativity in CTX binding to human leukemic T-cells and to rat heart myoblasts, which was then confirmed by Hill analysis, suggests that CTX may bind such cells differently than it binds fetal lung cells. Binding differences could underlie the striking difference in CTX susceptibility between a murine lymphoma cell line and murine T-cells [21]. Hill coefficients greater than 1.0 may reflect ligand binding to two or more interacting acceptor sites [6]. Such interaction could occur as previously inaccessible membrane sites become exposed due to sequential ligand binding [35]. Alternatively, ligand binding to multiple sites within a single subunit could result in increasing affinities [28], or interactions could occur within a multisubunit complex [1]. Elucidating the mechanism of CTX positive cooperativity will require further work.

The apparent dissociation constants observed for these two cell types (0.69 μM for CEM and 0.75 μM for heart cells) suggest only a moderate affinity of CTX for these membranes' binding molecules. Other well-studied systems, such as those involving the binding of neurotoxins, quinuclidinyl benzilate, or hormone peptides to purified receptors, exhibit K_Ds in the nanomolar range [e.g., 15, 26, 27, 39]. Often, low to moderate affinities can suggest that observed binding is not to a unique receptor [5]. The value for the K_D determined using kinetic and equilibrium binding data (0.76 μM) for CEM cells is not significantly different from that obtained using saturation data (0.69 μM). Using mouse heart cell membranes, Tonsing et al. [37] had similarly reported that CTX-$V^{II}1$ isolated from *Naja haje annulifera* had a K_D of 0.5 μM.

Although the same value of K_D for the CEM cells was obtained using two different experimental approaches, this was not the case for the heart cells. Dissociation data for the heart cells resulted in a plot from which the k_{-1} could not be derived. From the dissociation curves for both cell types, a rapid initial release of labeled CTX was observed. In the case of the heart cells, however, this rapid dissociation was followed by a rise in the fraction bound. While the basis for this rebound phenomenon remains to be determined, it is possible that following the binding of CTX to a heart cell, the membrane is so extensively disrupted by the addition of a 100-fold excess of nonradiolabeled toxin that cytoplasmic constituents become available for subsequent CTX

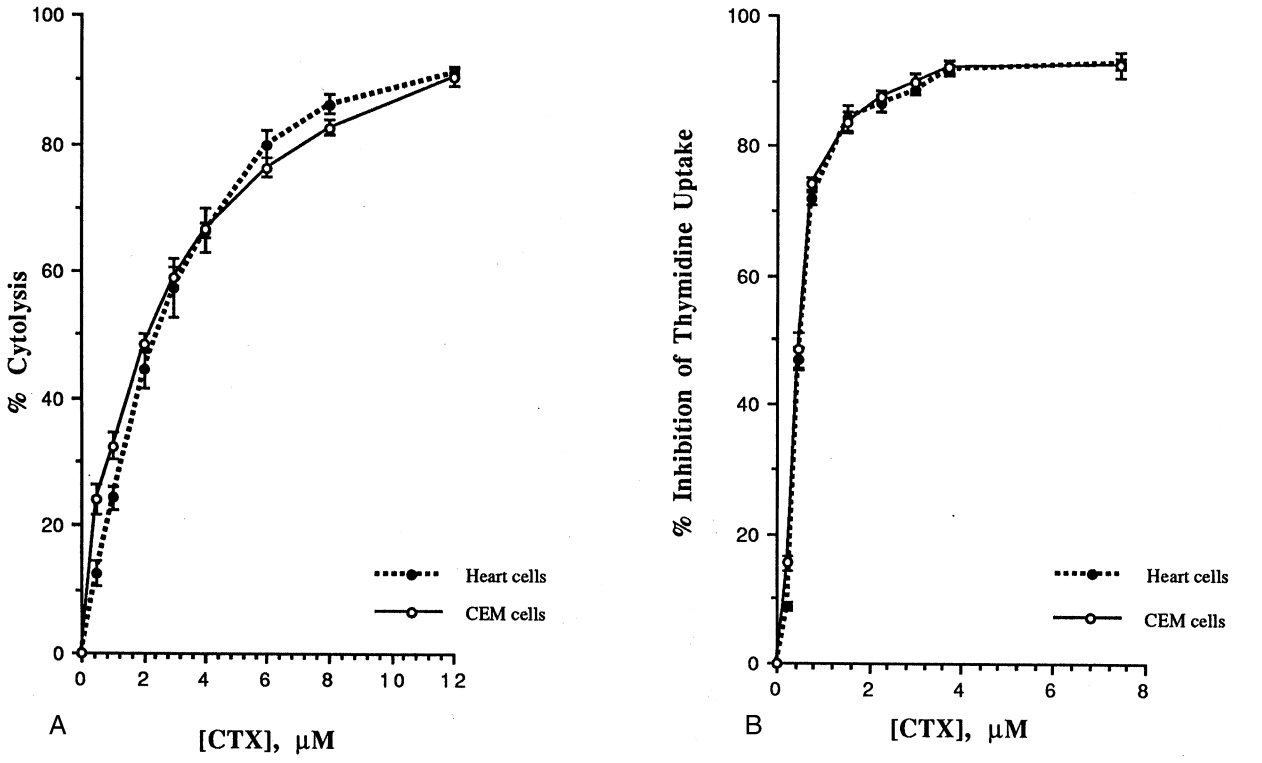

Fig. 2. Loss of cell viability as a function of cytotoxin concentration. Values shown represent the means ± SEM of three separate experiments, each performed in duplicate. (*A*) % cytolysis as determined by MTT incorporation assay; (*B*) % inhibition of thymidine uptake. ● = heart cells; ○ CEM cells

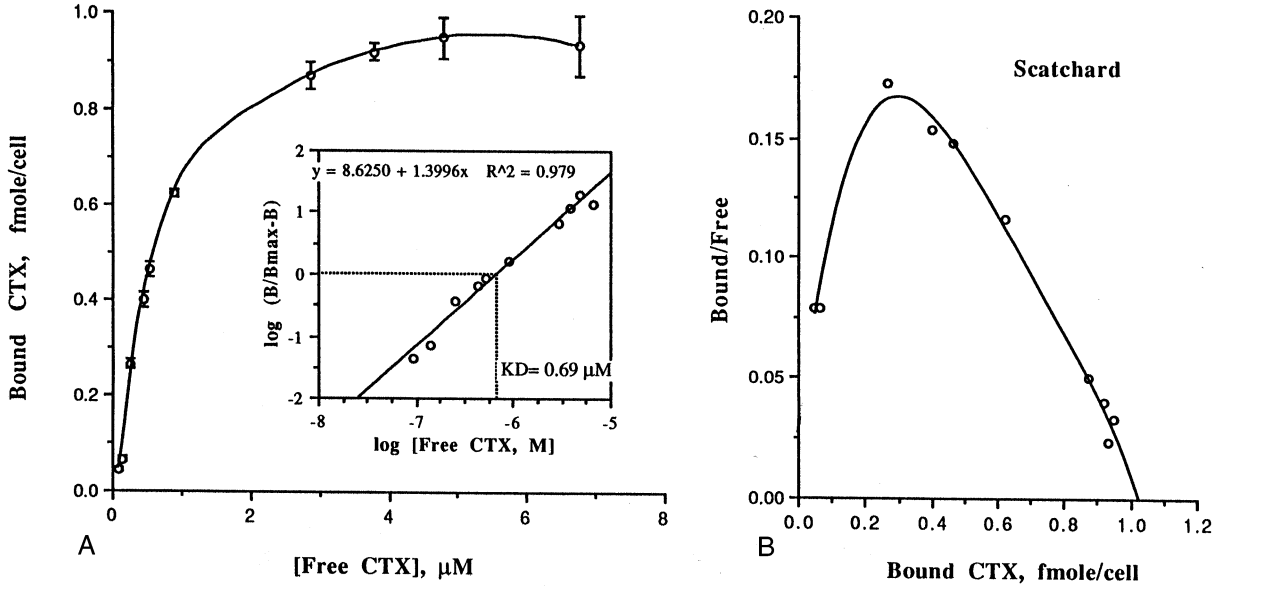

Fig. 3. Binding of labeled toxin to CEM cells. (*A*) Saturation binding; values shown represent the means ± SEM of three separate experiments, each performed in triplicate. Inset figure shows a Hill analysis of the data. (*B*) Rosenthal/Scatchard analysis of the data.

binding. The slightly greater value of the Hill coefficient for CTX binding to heart cells could support such an interpretation.

Coinciding with this relatively low binding affinity is an apparently high number of putative binding sites, which also suggests that binding may not be to a unique protein receptor in the plasma membrane of the target cell. It is possible that CTX-III binds directly to phos-

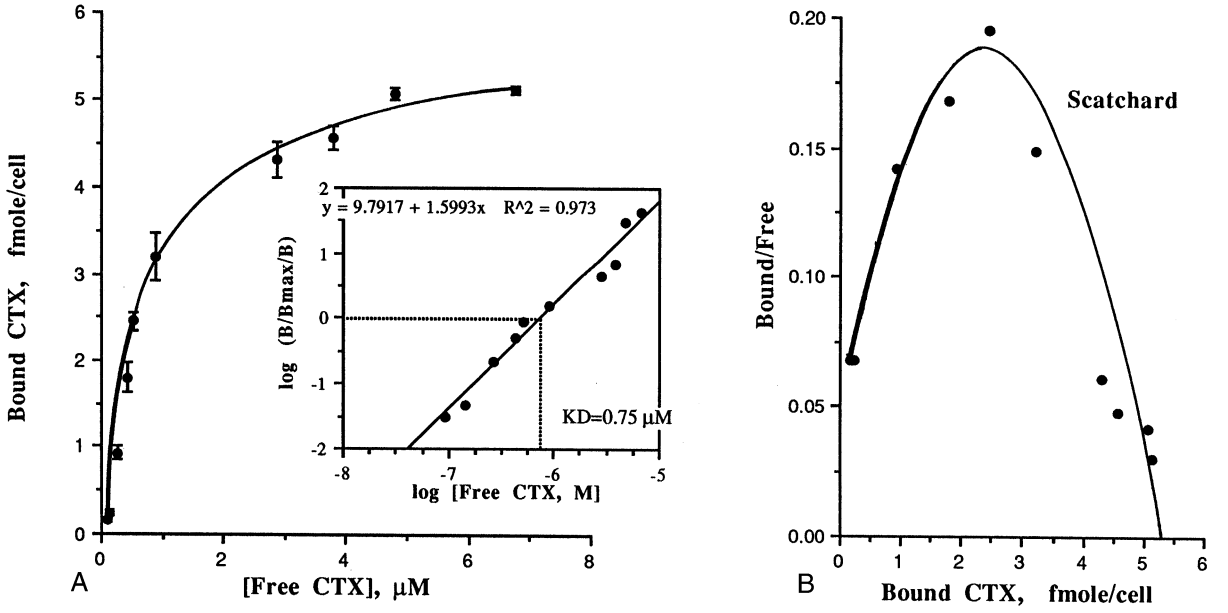

Fig. 4. Binding of labeled toxin to heart cells. (*A*) Saturation binding; values shown represent the means ± SEM of three separate experiments, each performed in triplicate. Inset figure shows a Hill analysis of the data. (*B*) Rosenthal/Scatchard analysis of the data.

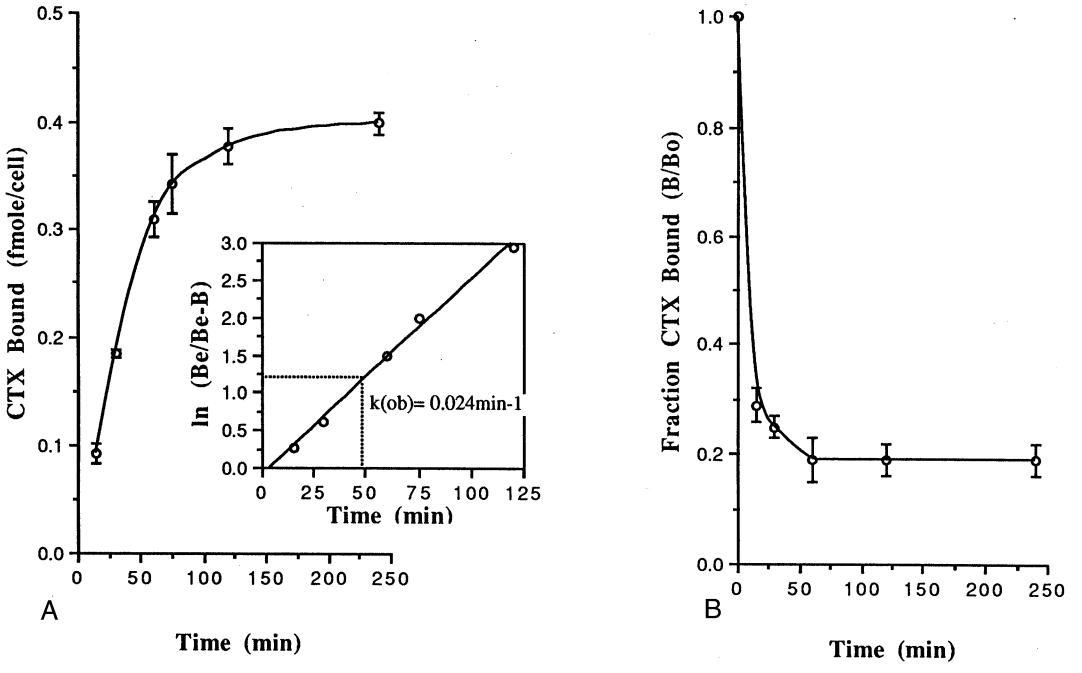

Fig. 5. Kinetics of binding of toxin to CEM cells. (*A*) Association binding as a function of time; values shown represent the means ± SEM of two individual experiments performed in triplicate. Inset figure presents the natural logarithm of the fraction bound as a function of time. (*B*) Dissociation of bound radiolabeled toxin as a function of time; values represent the means ± SEM of two individual experiments performed in triplicate.

pholipids in the membrane bilayer (as suggested for *Naja nigricollis* toxin γ [3, 14, 33]). This could occur as a result of salt bridge formation between cationic sites on CTX and the phosphate and/or carboxylic groups of the phospholipids, which is then further stabilized by hydrophobic interactions between lipid fatty acids and the apolar regions of CTX. Dufourcq and Faucon [11] as well as Vincent et al. [38], investigated the interaction of CTX isolated from *Naja mossambica mossambica* with lipid vesicles and reported that negative phospholipids such as phosphatidylserine, phosphotidylinositol and phosphatidic acid readily bound CTX with an association con-

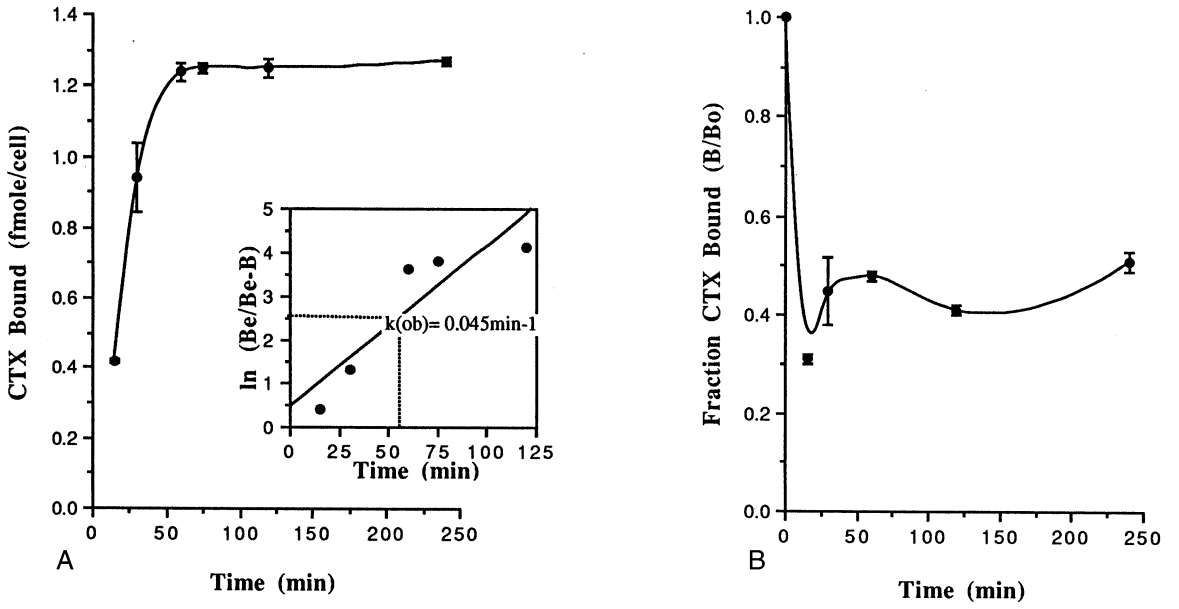

Fig. 6. Kinetics of binding of toxin to heart cells. (*A*) Association binding as a function of time; values shown represent the means ± SEM of two individual experiments performed in triplicate. Inset figure presents the natural logarithm of the fraction bound as a function of time. (*B*) Dissociation of bound radiolabeled toxin as a function of time; values represent the means ± SEM of two individual experiments performed in triplicate.

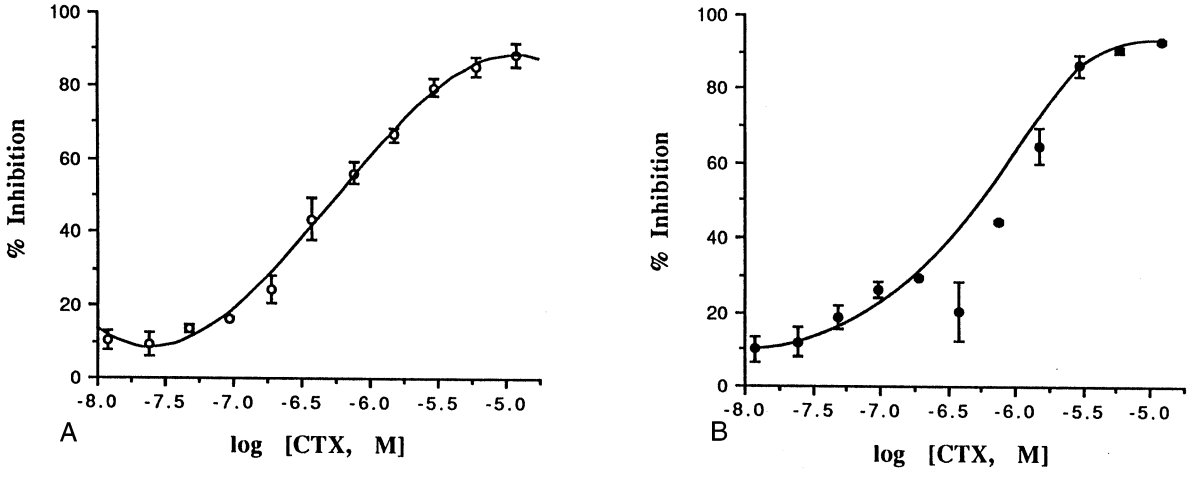

Fig. 7. Inhibition of binding of labeled toxin by unlabeled toxin. Values shown represent the means ± SEM of two individual experiments, each performed in triplicate. ● = heart cells; ○ = CEM cells

stant $>10^6$ M^{-1} [11], which is similar to the submicromolar dissociation constant we found. Takechi et al. [36] reported that negatively charged but not neutral exogenous phospholipid was able to reduce CTX′ cytolytic activity. Indirect support for a phospholipid binding site also comes from the report of Tonsing et al. [37], who indicated that trypsinization of target cells did not reduce binding of radiolabeled CTX. Chien et al. [9], who investigated the interaction of CTX variants with zwitterionic phospholipid dispersions, proposed that CTX could bind to two distinct targets: membrane phospholipids plus an inwardly rectifying K^+ channel. Nevertheless, only 15% or less of the lipids in several types of biological membranes carry a net negative charge at physiological pH, and the majority of these lipids are located in the inner leaflet of the bilayer, where they would not be directly accessible for CTX binding [12, 19]. Moreover, unless the outer lipid constituents of normal B-cells, T-cells, and macrophages, as well as those in leukemic B-cells, T-cells, and lymphoma cells differ significantly, CTX selectivity for normal and leukemic T-lymphocytes [21] suggests the existence of unique binding sites. A 59

Table 1. Binding and lytic parameters of CTX III for CEM cells and heart cells

	CEM cells	Heart cells
Determined by:		
	K_D, (μM)	K_D, (μM)
Saturation	0.69 ± 0.04	0.75 ± 0.02
Inhibition	0.65 ± 0.06	0.79 ± 0.00
Kinetic analysis	0.76 ± 0.07	not determined
Average K_D	0.70 ± 0.06	0.77 ± 0.03
Hill coefficient	1.35 ± 0.04	1.59 ± 0.03
B_{max}	1.04 ± 0.02 fmole/cell	5.20 ± 0.03 fmole/cell
Toxicity	EC_{50} (μM)	EC_{50}, (μM)
MTT oxidation	2.20 ± 0.16	2.40 ± 0.24
Thymidine uptake	0.5	0.5

kDa CTX-binding protein in membranes obtained from cockroach heart has been reported [25]. It is uncertain, however, whether this protein was located on the intracellular or the extracellular face of the membrane, and whether or not it was simply a cytoplasmic protein which became available upon cell lysis.

We suggest that binding mechanisms are likely to be similar for the two types of cells here investigated. In addition, the susceptibilities of both cell types to the lytic action of the toxin appear to be similar. A model in which CTX first binds a surface acceptor molecule, and then subsequently inserts itself into the lipid bilayer seems to be the most consistent with our experimental data. Whether such an acceptor molecule is protein or phospholipid remains to be established.

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