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The Effects of Inhibitors Upon Pore Formation by Diphtheria Toxin and Diphtheria Toxin T Domain

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Abstract. The formation of pores by membrane-inserted diphtheria toxin is closely linked to the translocation of its catalytic chain across membranes. In this report a number of aromatic polyanionic molecules were identified that inhibit toxin-induced leakage of molecules from model membrane vesicles. One inhibitor, Cibacron blue, totally blocked pore formation. Aniline blue and Fast Green decreased the size of the molecule released by a given concentration of toxin. Amaranth appeared to reduce the maximal amount of leakage, without greatly affecting the size of the molecule released at a given toxin concentration. Finally, Ponceau S and Cibacron brilliant red appeared to exhibit a mixture of these various types of inhibition. The inhibitors neither prevented the conformational transition of the toxin to form a hydrophobic state at low pH, nor (with the exception of Cibacron Brilliant Red) appeared to strongly inhibit toxin binding to model membranes. Additional experiments showed release of trapped materials from model membranes by isolated T domain of the toxin was similar to that by whole toxin. The effects of inhibitors on T domain induced release was also similar to that they have on whole toxin. Therefore, it is likely that the inhibition of pore formation by whole toxin involves inhibitor interaction with the T domain. The inhibitors identified in this study may be helpful for development of agents that interfere with toxin action in vivo.

Key words: Fluorescence quenching — Membrane insertion — Lipid-protein interaction — Model membranes

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Introduction

After receptor-mediated endocytosis diphtheria toxin is transported to endosomes. The acidification of the endosomal lumen triggers a pH-dependent conformational change within the toxin molecule [18, 19]. This allows the toxin to penetrate the endosomal membrane, and results in the translocation of its A chain into the cytoplasm.

It has long been known that diphtheria toxin has the ability to create pores in model membranes and cells at low pH [1, 7, 12, 14, 15, 24, 32]. However, the role of pore formation in the ability of the toxin to translocate its catalytic A chain across membranes remains uncertain [19]. Several studies have shown that mutations preventing pore formation by the toxin also prevent translocation [8, 28]. However, it is unclear whether the behavior of such mutants reflects a direct role of pore formation in the translocation process or a lesion in proper membrane insertion that prevents both pore formation and translocation. Therefore, new approaches to studying the role of pore formation in toxin action would be valuable.

In the course of studies of pore formation by diphtheria toxin it appeared that the anionic, aromatic Cascade Blue dye molecule we used to assay pore activity might be a weak inhibitor of pore formation. Although we could not confirm this in later studies, it led us to identify other aromatic, anionic compounds which strongly inhibit pore formation by the toxin. Inhibitors of pore formation by bacterial toxins could have practical biomedical applications, and lead to a better understanding of role of toxin-induced pore formation in the translocation of the catalytic domain of the toxin into cells.

Materials and Methods

Materials

Anti-Cascade Blue rabbit IgG (H + L fractions) antibody (Ab) (2.5–2.8 mg/ml stock solution), Cascade Blue (CB) labeled dextrans (molecular weight 3 kD, 10 kD, 70 kD), the free Cascade Blue probe 8-methoxypyrenetrisulfonic acid (MPT; molecular weight 538), N-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine-PE), were purchased from Molecular Probes (Eugene, OR). The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DOPG) were from Avanti Polar Lipids. Triton X-114, N-oleoyl-DL-Trp ethyl ester (oleoyl-Trp), amaranth, 2-[5,6-Bis(4-sulfophenyl)-1,2,4-triazin-3-yl]-4-(4-sulfophenyl) pyridine trisodium salt (BSTP), Cibacron brilliant red 3B-A, Ponceau S, and fast green FGF were purchased from Aldrich Chemical. Cibacron Blue was purchased from Sigma Chemical.

Partially purified diphtheria toxin was purchased from Connaught Laboratories (Ontario, Canada) and both nucleotide-free and nucleotide-bound monomers were further purified as previously described [5, 21]. (Because the bound ligand ApUp does not bind tightly at low pH, the behavior of bound and free toxin in the type of experiments performed in this study are similar [3]). The toxin was stored in 15 mM Tris-Cl, 150 mM NaCl, pH 7.5, at 4°C. An *E. coli* strain expressing the isolated transmembrane domain (T domain) of diphtheria toxin was a gift from the lab of Dr. R. John Collier. The T domain was purified as described previously [33]. Purified T domain was stored in 15 mM Tris-Cl, 150 mM NaCl, pH 8.0 (TBS), at 4°C. The purity of the whole toxin and T domain was confirmed on silver-stained SDS polyacrylamide gels. CB-labeled dextrans were purified as described in the accompanying report, and Triton X-114 was precondensed as previously described [4].

Fluorescence Measurements

Fluorescence emission spectra and intensities were measured on a Spex 212 Fluorolog fluorimeter operating in ratio mode, and using, unless otherwise noted, a 10 mm excitation pathlength, 4 mm emission pathlength semi-micro quartz cuvette. Also unless otherwise noted, fluorescence was measured in either PBS (10 mM potassium PO_4, 150 mM NaCl, pH 7.2) or in Na acetate/NaCl buffer (0.1 M acetate, 0.15 M NaCl, pH 4.5). All experiments were performed at room temperature. For all fluorescence experiments background samples were prepared without fluorophore, and where the background fluorescence was significant, it was subtracted to yield the final values.

Measurement of CB and MPT fluorescence intensity was performed at excitation and emission maxima of 385 nm and 417 nm, respectively, for the CB-dextrans and 405 nm and 433 nm, respectively, for MPT (emission maxima for these compounds was the same at both pH 4.5 and pH 7.0). For most experiments with whole toxin 2.5 mm slits were used. In experiments involving the T domain, or inhibitors, the slits were changed to 1.0 mm in the excitation beam and 5.0 mm in the emission beam.

Protein (Trp) fluorescence and oleoyl-Trp fluorescence were monitored with excitation at 280 nm and emission at 335 nm, using 2.5–3 mm slits.

Measurement of the Release of Entrapped Dextrans or MPT by Whole Toxin and Isolated T Domain

Large unilamellar vesicles (LUV) composed of 20% DOPG/80% DOPC (mol/mol) and containing entrapped CB-dextrans or MPT were prepared by octyl glucoside dialysis basically as described in the preceding paper except that the initial concentration of lipid prior to dialysis was 10–30 mM, so that the final lipid concentration in the vesicles obtained was in the range of 4–12 mM. The dependence of the release of entrapped material by whole toxin in the presence of inhibitors was measured similarly as described in the accompanying paper. An aliquot (9 μl–22 μl) of the vesicles was diluted to 150 μl with acetate/NaCl pH 4.5 buffer. Then a 5.5 μl aliquot of the inhibitor (10 μM final concentration) was added to the samples. Next the desired aliquot of toxin was added and after a 30 min incubation the sample was diluted to 550 μl with acetate/NaCl pH 4.5 buffer to yield a final lipid concentration of 200 μM. Fluorescence of the entrapped material was measured and then anti-CB antibody was then added to the samples in sufficient quantities (an aliquot of the stock antibody solution of no more than 5 μl) to obtain maximal (generally about 80%) quenching upon release of all of the entrapped material.

Release *vs.* T domain concentration experiments were performed similarly diluting the aliquot of the 4–12 mM stock solution of DOPG/DOPC vesicles containing entrapped MPT, or CB-dextrans to 150 μl with acetate/NaCl pH 4.5 buffer. When desired, a 5.5 μl aliquot of the inhibitor (10 μM final concentration) was added to the samples and then a 2.5 μl–15 μl aliquot was then added from 0.01 μg/μl–1.0 μg/μl T domain stock solutions in TBS to obtain the desired T domain concentration. The sample volume was then increased to 550 μl with acetate/NaCl pH 4.5, and after a 30 min incubation the fluorescence was measured before and after addition of an aliquot of antibody sufficient to bind all the released Cascade Blue. The % of maximal quenching (the quenching after all entrapped material is released by octyl glucoside) was calculated as described in the accompanying report.

The kinetics of the release of entrapped materials by the T domain were measured by adding 10 μg T domain (10 μl of a 1 mg/ml stock solution) to a 540 μl sample containing 200 μM DOPG/DOPC vesicles in acetate/NaCl pH 4.5 buffer and sufficient antibody to obtain maximal quenching upon release of the entrapped MPT or CB-dextran. Fluorescence was read before T domain addition, and after T domain was added and the sample was vortexed for 20 sec, fluorescence was measured as a function of time.

Experiments varying lipid/protein ratio at a constant T domain concentration were performed by diluting aliquots of a 5–12 mM stock of DOPG/DOPC vesicles containing entrapped 10 KD CB-dextran to 150 μl with acetate/NaCl pH 4.5 buffer. An aliquot of T domain (1 μl from a 1 μg/μl stock) was added to the sample and incubated for 45 min. The sample volume was brought up to 550 μl with acetate/NaCl pH 4.5 buffer and the fluorescence was measured before and 30 min after an addition of an aliquot of antibody (1 μl–10 μl) sufficient to obtain maximal quenching. The final lipid concentrations in the samples were 0, 13.3, 20, 40, 80, 200, 400, and 800 μM.

T domain titration experiments were performed by adding an aliquot (9 μl–22 μl) of a 5–12 mM stock solution of DOPG/DOPC vesicles containing entrapped 10 KD CB-dextran to a tube containing sufficient acetate/NaCl buffer pH 4.5 to bring the volume to 550 μl (200 μM lipid). Antibody was added to the sample at sufficient levels to obtain maximum quenching of released materials. After measurement of Cascade blue fluorescence successive 1 μl aliquots of T domain (from a 1 mg/ml stock solution) were added, incubated for 3 min, and the fluorescence was remeasured.

Absorbance of Inhibitors

Stock solutions containing 1 or 10 mM inhibitor were prepared in PBS. To estimate whether inner filter effects would make fluorescence intensity measurements difficult due to inhibitor absorbance, and whether

Table. Absorbance of 10 μM Solutions of Potential Inhibitors at pH 4.5.

	Absorbance at			
	280 nm	335 nm	385 nm	417 nm
Molecule				
Cibacron Red	.40	.082	.040	.027
Aniline Blue	.019	.017	.002	.001
Ponceau S	.13	.13	.10	.091
Amaranth	.15	.11	.052	.060
Fast Green	.089	.033	.051	.12
Cibacron Blue	.24	.10	.050	.028
BSTP	nd	.07	.004	<.01

nd = not determined.

Trp to inhibitor energy transfer was possible, the absorbance of each inhibitor was measured after dilution to 10 μM with acetate/NaCl pH 4.5 buffer (Table 1). Aliquots from the stock solutions were used in subsequent experiments.

Fluorescence Quenching Assay of Inhibitor Binding to Membrane-Bound Toxin

The ability of the inhibitors to bind to membrane bound whole toxin was assayed by adding 10 μl of a 1 mg/ml stock solution of bound monomer toxin to 540 μl acetate/NaCl pH 4.5 buffer containing 200 μM 20% DOPG/80% DOPC LUVs (prepared by freeze-thawing in dry ice/acetone, followed by extrusion through 21 extrusions through 100 nm polycarbonate filters using a Liposofast miniextruder (Avestin, Ottawa, Canada)). Under these conditions there is almost complete binding of the toxin to the vesicles [6, 11]. After transfer of the sample to a cuvette, protein fluorescence was measured. Fluorescence was remeasured after each of a series of additions of 1.1 μl aliquots of inhibitor mixed with the sample by briefly vortexing.

The effect of inhibitors on tryptophan fluorescence via an inner filter effect was measured using a sample containing a 2.5 μl aliquot of free tryptophan (from a 1 mM stock solution) diluted to 550 μl with pH 4.5 acetate/NaCl buffer. After measuring fluorescence, the inhibitors were titrated into the sample in 1.1 μl aliquots, measuring fluorescence after each aliquot. The intensity of fluorescence in a toxin or oleoyl-Trp sample which would have been obtained in the sample in the absence of an inner filter effect ($F_{corrected}$) was calculated using the equation $F_{corrected} = F_{measured}\,(F_{Trp}/F_{Trp+inhibitor})$. At the highest concentration of the most strongly absorbing inhibitors (10 μM) there was up to 50% loss of fluorescence due to the inner filter effect.

Fluorescence Quenching of Oleoyl-Trp

To distinguish inhibitor binding to the vesicles from inhibitor binding directly to toxin, the degree of inhibitor-induced quenching of the fluorescence of a vesicle bound Trp analogue was measured. LUVs containing 19.95% DOPG/79.95% DOPC and 0.1% oleoyl-Trp (mol/mol) were prepared at a concentration of 40 mM in PBS by extrusion (*see above*). Samples contained a 3.26 μl aliquot from a 40 mM stock solution of the oleoyl-Trp containing vesicles diluted to 550 μl with acetate/NaCl pH 4.5 buffer (final concentration of 200 μM lipid). Aliquots increasing inhibitor concentration in 2 μM steps were added, followed by a 2 min incubation after which the fluorescence was measured. The final binding curves were corrected for the amount of inter filter effect as for the toxin-containing sample (*see above*).

Triton X-114 Phase Partition Assay

Triton X-114 phase partition assay was performed using the method of Bordier [4]. Each sample contained 480 μl of various pH buffers. Buffers at pH 6.0 and above were composed of 0.1 M potassium phosphate and 0.15 M sodium chloride. Buffers at pH 5.5 and below contained 0.1 M sodium acetate and 0.15 M sodium chloride. The buffers were kept on ice. To each sample 50 μl of precondensed Triton X-114, 5.5 μl from the 1 mM stock solution of each inhibitor, and then a 20 μl aliquot of a 1 mg/ml stock solution of bound monomer toxin was added. The samples were then vigorously vortexed and then incubated on ice for 5 min. After incubation at 37°C for 5 min they were then subjected to a 10 min spin at room temperature using a Fisher model 235A table top microcentrifuge at 13,000 × g. A 500 μl aliquot containing the supernatant was removed from the samples and saved for analysis. The detergent containing pellets were washed by the addition of 500 μl cold buffer to each sample and repeating the incubation and centrifugation steps. A 500 μl aliquot of the supernatant was removed and discarded, and the pellets resuspended with 500 μl of the appropriate buffer. The resuspended pellets were kept on ice (to keep the detergent soluble). The samples were diluted 1:4 with 4× sample buffer and subjected to SDS-PAGE using Pharmacia pre-cast 10%–15% gradient gels and electrophoresis carried out as previously described [12]. The gels were visualized using silver staining.

Lipid-Binding Experiments

The ability of whole toxin and the isolated T domain to associate with lipid at low pH was measured using a density flotation assay. A solution of 10% sucrose was prepared by dissolving 1 g of sucrose in 10 ml of 0.1 M Na acetate pH 4.5/0.15 M NaCl. A 21 mM stock solution of 20% DOPG/80% DOPC LUVs containing trace amounts of rhodamine-PE were prepared by octyl glucoside dialysis (*see above*) except omitting the chromatography on Sepharose 4BCL. In one protocol, samples were prepared by placing 274 or (if no lipid was present) 287 μl of a 10% sucrose solution in pH 4.5 buffer into a Beckman Ultra Clear 8 × 20 mm tube (Beckman Instruments, Palo Alto, CA) containing 1.1 μl of a 10 mM stock solution of inhibitor. LUVs were added to the tube to a lipid concentration of 800 μM (11.2 μl) from the lipid stock. The samples were gently vortexed, then an aliquot containing 20 μg of toxin (from 1.5 mg/ml stock solutions) was added and mixed. In the second protocol, the inhibitors were only added after the addition of the toxin to the vesicles. In both protocols the final volume was 300 μl. The samples were incubated for 30 min and then subjected to ultracentrifugation in a Beckman Airfuge (set at 20 psi) for 30 min. The top layer of the sucrose was removed by skimming to remove as much of the lipid as possible. A total volume of 280 μl of the sucrose solution was removed from the sample in this way and this lipid-containing fraction was denoted the supernatant fraction. The remainder of the sample was considered the pellet fraction. After diluting the pellet to 300 μl, aliquots were removed from both the supernatant and pellet fractions, diluted 1:4 in 4× sample buffer, subjected to SDS-PAGE using Pharmacia 10–15% gradient phastsystem gels, and stained with Coomassie Blue. The rhodamine fluorescence in each sample was quantified by resuspending the remainder of both the supernatant and pellet fractions to about 600 μl with buffer and measuring the fluores-

cence at an excitation wavelength of 565 nm and an emission wavelength of 585 nm. The percent lipid remaining in each fraction was calculated from the ratio: [rhodamine fluorescence in the sample]/[total rhodamine fluorescence]. Experiments in which the inhibitor was added after toxin addition were performed in the same manner as described above except that the inhibitor was added to the sample after the 30 min incubation of the toxin with the lipid vesicles in the 10% sucrose solution. Once the inhibitor was added, the sample was incubated for a further 30 min before being subjected to the analysis described above.

Results

Inhibitors Bind to Membrane-Inserted Diphtheria Toxin at Low pH

We examined the ability of a number of aromatic, anionic molecules (several of which were already known to interact with some biomolecules [16, 20, 22, 29]) to prevent the release of vesicle-entrapped materials by diphtheria toxin. The association of these molecules with diphtheria toxin was examined under conditions where the toxin is membrane-inserted (20% DOPG/80% DOPC vesicles at pH 4.5) [6, 11]. As the molecules chosen were all dyes with absorbance overlapping Trp fluorescence emission to some degree (Table 1), they can act as energy transfer acceptors of Trp fluorescence. Therefore, binding to toxin was assayed by determining the degree to which they quenched the tryptophan fluorescence of toxin molecules[1].

As shown in Fig. 1 all of the inhibitors gave saturable quenching that was half-maximal in the 1–5 μM range[2]. No quenching was observed in a control titration with buffer alone. In an additional control, the quenching of the hydrophobic probe oleoyl-Trp by these potential inhibitors was measured to see if quenching was due to the binding of the inhibitors to the vesicles (in which case they should be able to quench membrane-inserted oleoyl-Trp) rather than directly to the toxin (in which case there should be no quenching of oleoyl-Trp)[3]. With the exception of Cibacron Blue no significant quenching of oleoyl-Trp by the inhibitors was found (Fig. 1). This indicates and that except for Cibacron Blue the dye-induced quenching of fluorescence is due to their binding toxin rather than to the vesicles. (In agreement with this result Cibacron Blue was the only dye that bound to vesicles as judged visually in centrifugation experiments (*data not shown*).) Even Cibacron Blue appears to directly bind toxin as shown by its stronger quenching of toxin fluorescence relative to that of oleoyl-Trp[4]. Therefore, the quenching of toxin fluorescence by the potential inhibitors appears to reflect their direct binding to protein in all cases.

Inhibition of Diphtheria Toxin Pore Formation

The effect of potential inhibitors on pore formation was also examined. Near-saturating concentrations (10 μM) of each potential inhibitor was added to vesicles loaded with fluorescent leakage markers (MPT or Cascade-Blue labeled dextran). Diphtheria toxin was then added, again using conditions in which it rapidly and efficiently inserts into the membranes (20% DOPG/80% DOPC (mol/mol) pH 4.5 [6, 11]). The release of markers was measured by the quenching induced when they became bound to externally added anti-CB antibodies[5].

In agreement with the accompanying report, the level of release was generally dependent on both toxin concentration and dependent on the size of the trapped molecule, with larger dextrans released only at higher toxin concentrations. In the presence of the potential inhibitors different degrees of inhibition were detected (Fig. 2). At one extreme, the molecule BSTP had little or no effect on dextran release. At the other extreme, complete inhibition was observed for Cibacron Blue. The other molecules examined gave intermediate degrees of inhibition. Aniline Blue, and more weakly, Fast Green increased the concentration of toxin need to obtain dextran release. For example, the concentration of toxin yielding 50% release of the 3 kD CB-dextran showed a 5–10-fold increase in the presence of these inhibitors. An increased toxin concentration was also needed to release MPT and the 10 kD CB-dextran. In contrast, amaranth reduced the amount of maximal leakage by a third without affecting the concentration of toxin needed to

[1] We could not estimate the binding affinity of toxin for Cibacron Red because of the strong absorbance on Cibacron Red in the ultraviolet, which interferes with measurement of Trp fluorescence via the inner filter effect.

[2] Differences in the maximal amount of quenching are a function of several factors, including the degree of spectral overlap with Trp fluorescence, the number of inhibitor molecules bound per toxin molecule, and the location of their binding site on the toxin. Therefore, they cannot be interpreted easily. It is also possible that quenching mechanisms other than dipole-dipole type energy transfer is involved in the quenching process for some of the inhibitors.

[3] Oleoyl-Trp should be very hydrophobic, and tightly bound to vesicles under our experimental conditions [17]. This was confirmed by examining the dependence of oleoyl-Trp fluorescence on lipid concentration when DOPG/DOPC vesicles were titrated into a solution of oleoyl-Trp. There was an increase of oleoyl-Trp fluorescence intensity that was half-maximal at about 10 μM lipid.

[4] Since Cibacron Blue is highly charged, and unlikely to penetrate the bilayer, the quenching of oleoyl-Trp is likely to involve interaction of oleoyl-Trp molecules in the outer leaflet of the bilayer with Cibacron Blue molecules that are also on the outer leaflet. Cibacron Blue quenching of the toxin is also likely to only involve Cibacron Blue molecules on the outside of the membrane because it blocks pore formation.

[5] The degree of inhibition of MPT release may be influenced slightly by the degree to which MPT itself binds to the toxin and inhibits its own release.

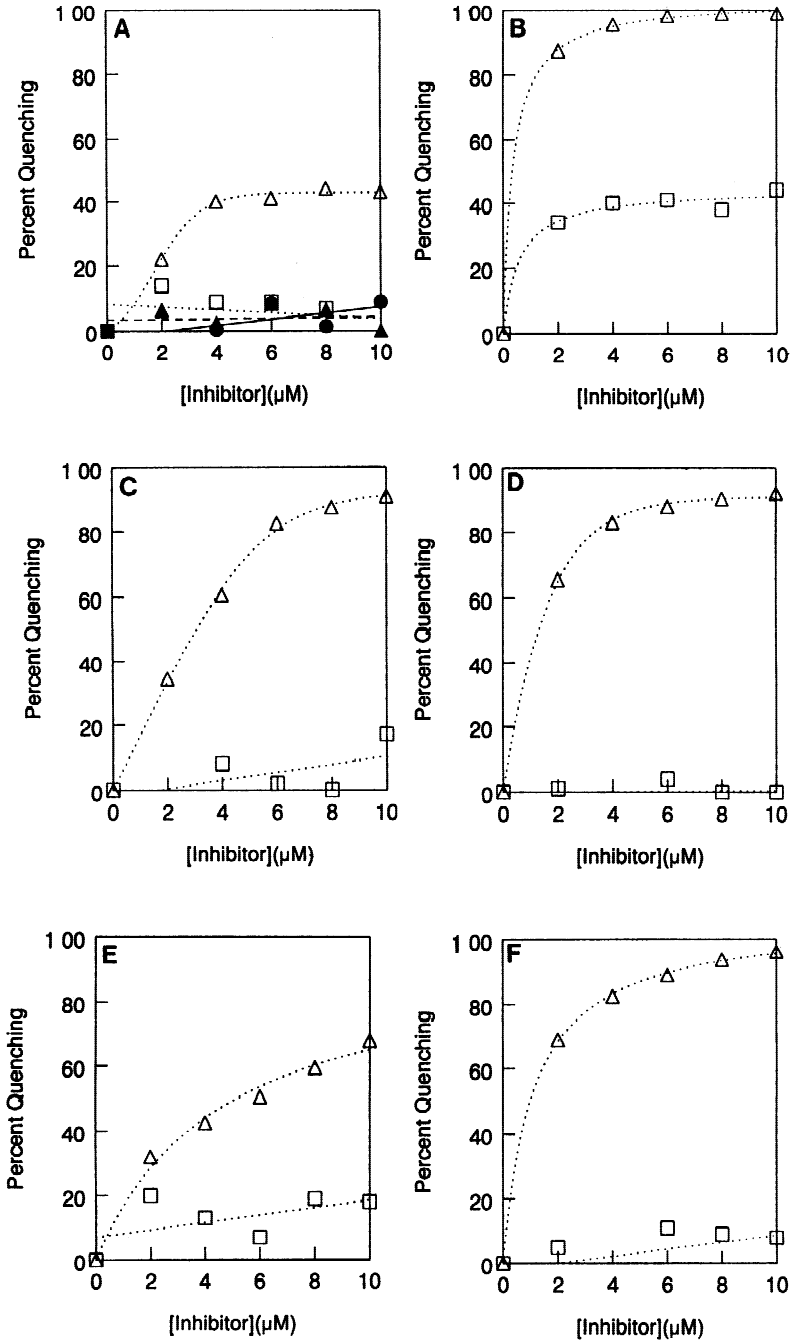

Fig. 1. Quenching of the fluorescence of (open triangles) whole diphtheria toxin and (squares) oleoyl-Trp by: (*A*) Amaranth, (*B*) Cibacron Blue, (*C*) BSTP, (*D*) Ponceau S, (*E*) Fast Green, and (*F*) Aniline Blue at pH 4.5. Samples contained 18 μg/ml toxin or 2.6 μM oleoyl-Trp incorporated into 200 μM 20% DOPG/80% DOPC vesicles at pH 4.5. Filled symbols in (*A*) show mock (buffer) treated whole toxin and oleoyl-Trp.

give half-maximal release. Ponceau S and Cibacron Red showed a mixed behavior, involving both a marked reduction in the level of maximal leakage, plus an increase in the amount of toxin needed for 50% maximal leakage.

Control experiments with Cascade Blue and antibody in solution in the presence of the inhibitors gave maximal quenching (70–80%) demonstrating that the reduced degree of maximal quenching observed for some of the inhibitors was not due to their having an effect on Cascade Blue binding to antibody (*data not shown*). It should also be noted that the absorbance of the inhibitors was too weak to affect fluorescence intensity via an inner filter effect (Table 1).

The Formation of a Hydrophobic State by Diphtheria Toxin is Largely Unaffected by Inhibitors

There are several possible mechanisms by which inhibitors could prevent toxin pore action. One is that they

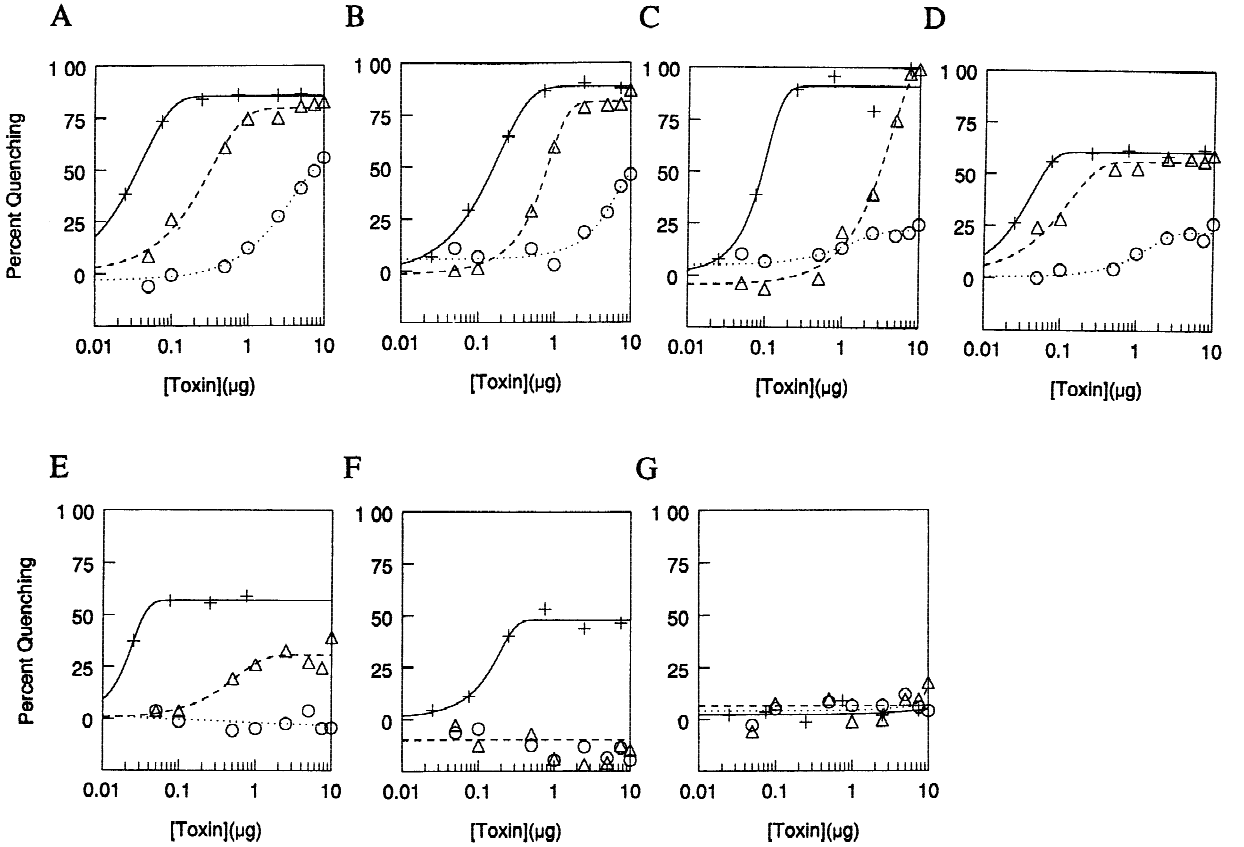

Fig. 2. Release of CB-labeled molecules from 200 μM 20% DOPG/80% DOPC vesicles at pH 4.5 as a function of the amount of toxin added. The percent (of maximal) quenching of CB fluorescence by external anti-CB antibody is shown for: MPT (pluses), 3 kD CB-dextran (triangles) and 10 kD CB-dextran (circles). Assays were performed in the presence of 10 μM: (*A*) BSTP, (*B*) Fast Green, (*C*) Aniline Blue, (*D*) Amaranth, (*E*) Cibacron Red, (*F*) Ponceau S, or (*G*) Cibacron Blue. Sample volume was 550 μl.

could affect the ability of the toxin to become hydrophobic and insert into membranes at low pH (which normally occurs at pH 5 and below [3]).

To examine this possibility, toxin hydrophobicity was measured as a function of pH using the Triton X-114 (TX-114) phase partition assay (Fig. 3). At 37°C TX-114 becomes insoluble and can be separated from the buffer together with detergent-bound hydrophobic proteins [4]. We previously found that this assay can be used to detect the conversion of the toxin to its hydrophobic, membrane penetrating form at low pH [23].

Figure 3 shows the partitioning of the toxin between the aqueous and TX-114 enriched detergent phase in the presence and absence of inhibitors as a function of pH. In all cases, the toxin is able to undergo the transition to the hydrophobic state at low pH. At most, there is small affect on the pH at which the toxin becomes hydrophobic in the presence of inhibitors (in the range pH 5–6). Nevertheless, in all cases the toxin is hydrophobic at pH 4.5. Therefore, the inhibitors do not affect pores by preventing the toxin from undergoing conversion to a hydrophobic state at low pH.

Effect of Inhibitors on Toxin Association with Membranes

Although the toxin becomes hydrophobic at low pH in the presence of the inhibitors, the possibility exists that these compounds inhibit toxin association with lipid bilayers. The degree of association of toxin with vesicles at low pH was examined using ultracentrifugation in 10% (w/v) sucrose. In this assay, the vesicles and vesicle bound toxin should remain in the supernatant, whereas the toxin that is unbound to the vesicles should aggregate and could pellet. (In the absence of inhibitors, toxin is totally bound to such vesicles at low pH [6].) These experiments were complicated by the fact that even under the best conditions we could identify only a fraction of the vesicles (and thus vesicle bound toxin) remained in the supernatant (even in the absence of inhibitor (not shown)). Furthermore, toxin pelleting in the absence of lipid was only observed when certain inhibitors were present.

Figure 4 shows the results of such centrifugation experiments in which the amount of protein in the pellet

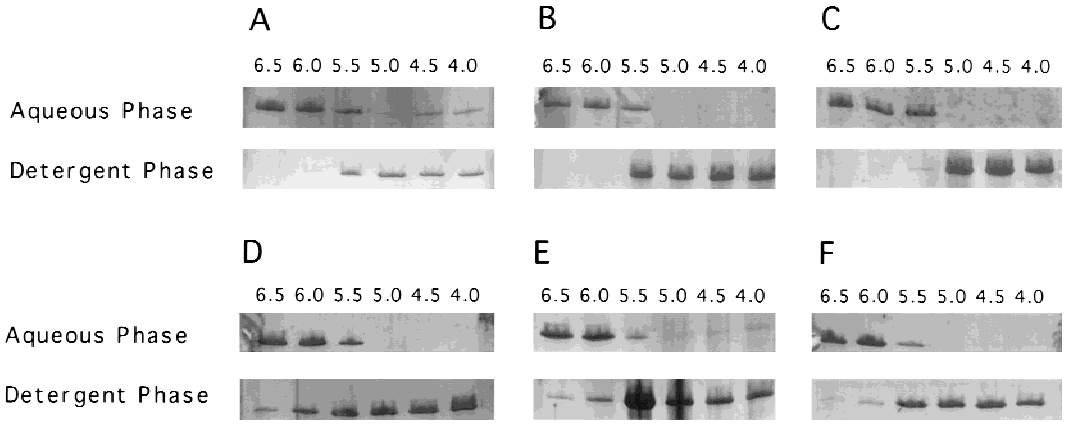

Fig. 3. Phase partitioning of diphtheria toxin into TX-114 as a function of pH. Represented are silver stained SDS polyacrylamide gels of toxin phase partitioning *vs.* pH for: (*A*) whole toxin alone, or for whole toxin with 10 μM: (*B*) Cibacron Blue, (*C*) Cibacron Red, (*D*) Aniline Blue, (*E*) Fast Green, or (*F*) Ponceau S.

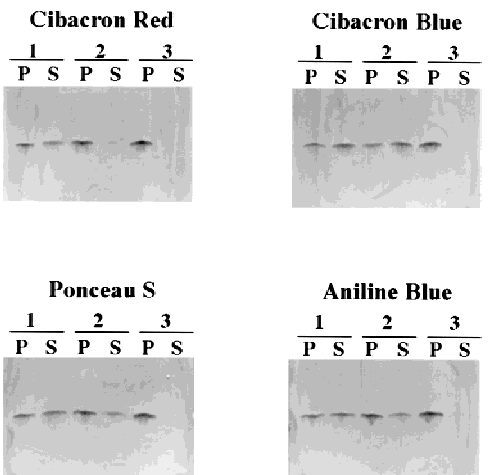

Fig. 4. Diphtheria toxin binding to lipid in the presence of inhibitors. Each sample contained 67 μg/ml whole toxin added to a solution containing 20% DOPG/80% DOPC vesicles and the compounds shown. The final lipid concentration was 800 μM and final inhibitor concentration 37 μM. The conditions examined were: 1, toxin and lipid mixed before the addition of inhibitor; 2, toxin and inhibitor mixed before the addition of lipid; 3, toxin and inhibitor mixed without lipid. P = pellet fraction; S = supernatant fraction. Under conditions 1 and 2, respectively, the % of lipid in supernatant was 50 and 88% for Cibacron Red, 64 and 55% for Cibacron blue, 44 and 74% for Ponceau S, and 47 and 66% for Aniline blue.

(P lanes) and supernatant (S lanes) was assessed by gel electrophoresis, and lipid location by the fluorescence of trace rhodamine labeled lipid (*see* Materials and Methods). These experiments were performed under conditions in which the inhibitor was added to the toxin after the addition of the lipid vesicles (condition 1), in which the inhibitor was added to the toxin prior to the addition of lipid vesicles (condition 2), and in which toxin and inhibitor were mixed in the absence of lipid (condition 3).

When inhibitors were added after toxin and vesicles were mixed, none had a strong effect on toxin binding to lipid (condition 1). Although half the protein was in the pellet, this can be accounted for by the inefficient (45–65%) pelleting of lipid in these samples (*see* figure legend) rather than dissociation from the bilayer. There seemed to be a more marked effect when inhibitor and toxin were mixed prior to addition of lipid (condition 2). Cibacron Red had an especially strong effect, completely inhibiting toxin binding to lipid under these conditions. Much smaller effects were observed for the other inhibitors, especially after again correcting for partial pelleting of the lipid. Nevertheless, it is possible that Ponceau S and Aniline blue also prevent binding to lipid to some degree.

These results raise the possibility that some inhibitors act by preventing toxin binding to lipid. To examine this possibility, inhibition experiments were repeated under conditions in which the toxin remains membrane bound, i.e., adding the inhibitor after toxin and lipid are mixed. To do this, anti-Cascade Blue antibodies were trapped in the vesicles, toxin was added, and after a 45 min incubation, the inhibitor. To initiate the assay, the Cascade Blue-labeled dextran was then added externally. Even with this protocol, under which the experiments above show the toxin remains membrane associated, strong inhibition was observed when Cibacron Red, Cibacron Blue, Ponceau S or Aniline blue was added (*data not shown*). Therefore, it does not appear likely that the effects of inhibitors on toxin binding to vesicles can explain most of the inhibition process.

Pore Formation by the T Domain of Diphtheria Toxin

To identify the site where inhibitors act, the pore-forming abilities of the T domain were examined. The T

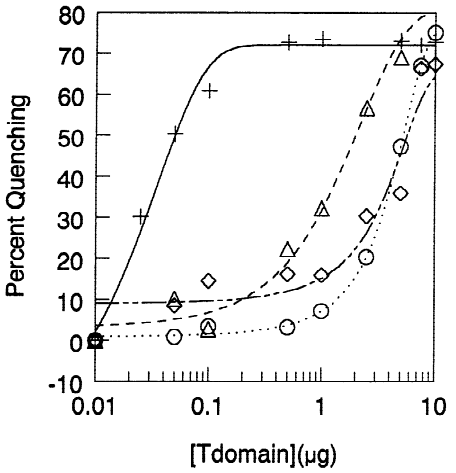

Fig. 5. Release of CB-labeled molecules from 200 μM 20% DOPG/80% DOPC vesicles as a function of the amount of T domain present. Release was measured for: MPT (pluses), 3 kD dextran (triangles), 10 kD dextran (circles) and 70 kD dextran (diamonds). Sample volume was 550 μl.

domain is believed to be responsible for the ability of the toxin to form pores [14, 27]. Figure 5 shows the dependence of leakage of markers induced by T domain at low pH. The dependence of pore properties on T domain concentration is similar to that by whole toxin, with larger dextrans able to leak out at high concentration. As in the case of whole toxin, leakage was rapid, and nearly complete after 30 min (*data not shown*). In addition, dextran release by the T domain was also similar to whole toxin in that it was dependent upon low pH, with release half maximal between pH 5.5 and 6. Finally, like whole toxin, a dependence of release on T domain/lipid ratio similar to that when protein concentration was varied in a series of individual samples (Fig. 5) was obtained when lipid concentration was varied instead of protein concentration, and when titrating a single sample of vesicles with a series of 1 μg aliquots of T domain (*data not shown*).

Quenching experiments demonstrated that the inhibitors bound T domain in a manner very similar to that of whole toxin[6]. Therefore, the effect of inhibitors on the release of vesicle entrapped MPT and CB-dextrans by T domain was examined. As shown in Fig. 6, a significant degree of inhibition was observed in several cases. Fast Green was a weak inhibitor, in agreement with the results obtained with whole toxin. It increased the amount of T domain necessary for maximal release of the 70 kD CB-dextran, but had little effect on the release of the small dextrans. Stronger inhibition was observed with Ponceau S, Cibacron Red and Cibacron Blue. Interestingly, inhibition by Cibacron Blue was incomplete, in contrast to the complete inhibition observed with whole toxin. Although the inhibitory behavior differs in some details, it appears that the properties of the pore formed by whole toxin and T domain are similar.

[6] The most notable differences were that addition of amaranth and BSTP resulted in significantly weaker quenching of Trp fluorescence than with whole toxin.

Discussion

Mechanism of the Inhibition of the Release of Vesicle Entrapped Materials

This report demonstrates that several aromatic sulfonic acid derivatives can prevent diphtheria toxin-induced release of markers from model membrane vesicles. Inhibition is likely to involve interaction of inhibitors with the T domain. This conclusion comes from the observations that: (i) the isolated T domain forms pores with properties similar to whole toxin (*see* Results and [14, 27]), and (ii) the inhibitors are able to inhibit both those pores formed by whole toxin and those formed by the isolated T domain. However, it is not necessarily true that inhibitors bind only to the T chain. For example, at neutral pH some Cibacron dyes can bind at the active site on the A chain [2, 25]. Therefore, it is possible that the effect of inhibitors on the A chain or even R domain of the toxin could influence pore formation in whole toxin.

There are several possibilities for the mechanism by which toxin-induced release of vesicle-entrapped substances may be blocked by inhibitors. In most cases, the inhibitors do not seem to act by affecting the step in which the toxin forms a hydrophobic, membrane-penetrating conformation at low pH. Instead, they may act by altering toxin conformation within membranes. In this regard, it is relevant that the T domain can form two conformations, only one of which appears to be deeply penetrating [13, 31]. Studies with mutant T domain molecules suggest that only the more deeply penetrating conformation is able to form pores [26]. Therefore, if inhibitors prevent the formation of the deep conformation, they could prevent pore formation. Another possibility is that the inhibitors prevent and/or alter the degree of toxin oligomerization, since toxin oligomerization also appears to be important to regulate pore properties (*see* accompanying report). Finally, it is possible the inhibitors simply physically block the pore by binding to the residues lining it.

Whatever the mechanism of inhibition, it must explain the reason(s) for the different types of inhibition observed with different inhibitors. Specifically, why do several inhibitors reduce the maximum level of release,

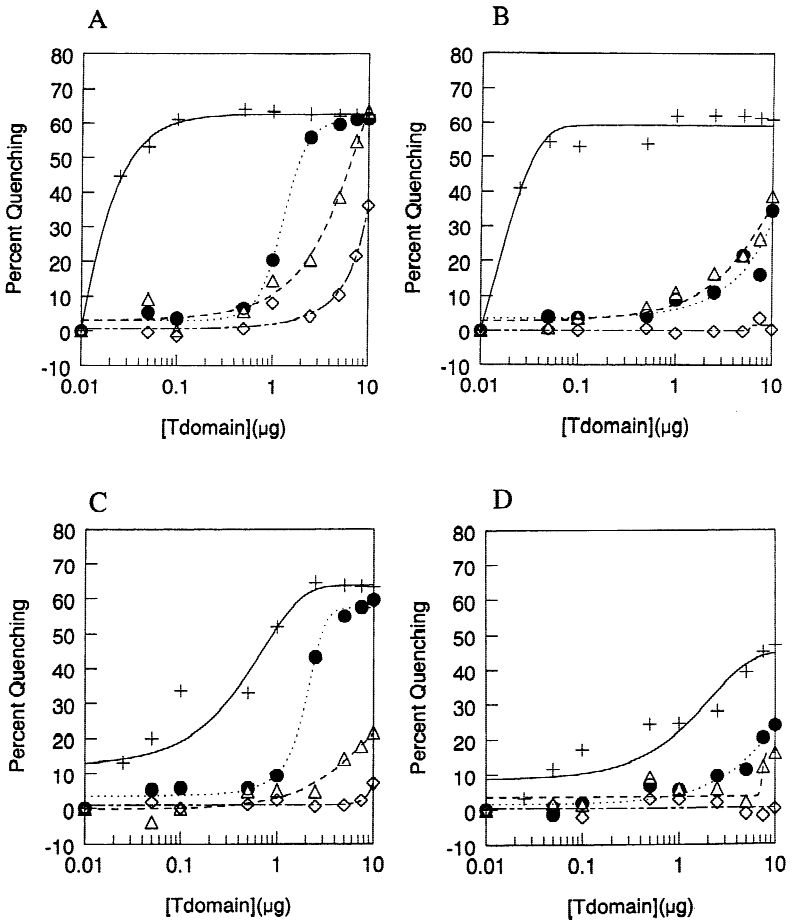

Fig. 6. Release of CB-labeled molecules for 200 μM 20% DOPG/80% DOPC vesicles as a function of the amount of T domain present performed in the presence of inhibitors. Release was measured for; MPT (pluses), 3 kD dextran (filled circles), 10 kD dextran (triangles) and 70 kD dextran (diamonds) in the presence of: (*A*) Fast Green; (*B*) Ponceau S: (*C*) Cibacron Blue; and (*D*) Cibacron Red. Sample volume was 550 μl.

whereas others only increase the concentration of protein needed to obtain maximal release? One factor that must be considered is the kinetics of the inhibition process. A reduced level of maximal release might occur if an inhibitor had the ability to totally block release, similar to Cibacron Blue, but only bound to the toxin slowly. In this case, there would be release of some materials prior to inhibitor binding[7]. Another possiblity is that the reduced release is an artifact of CB leakage prior to toxin addition and the method used to calculate % quenching (*see* accompanying paper). This is unlikely because we only observed such leakage in some vesicle preparations containing MPT, whereas the reduced release was detected for both MPT and the 3kD CB-dextran.

The requirement for an increased concentration of toxin to allow full dextran release might reflect the stoichiometry of inhibitors bound per toxin. In this case, inhibition could be relieved at excess toxin concentrations due to the decrease of the inhibitor/toxin ratio. Specifically, one example would be if the inhibitor only partially blocks pores, i.e., results in a narrowing of pore size, either directly (by binding to the pore lining residues), or indirectly (by reducing the degree of oligomerization). Since the size of the toxin pore increases as toxin concentration increases, higher toxin concentrations could counteract this narrowing.

Several additional questions concerning the inhibition process also need to be addressed by future studies. These include finding the number of inhibitors that bind to each toxin molecule, and the precise location(s) to which they bind. It will also be necessary to test a wider range of inhibitors in order to understand the relationship between chemical structure and inhibition. At present, it is hard to correlate the structural features of inhibitors with the degree to which they inhibit pore formation. For example, it is puzzling that BSTP inhibited release of dextran so weakly given the similarity of its structure to the other molecules tested. Furthermore, one report of the inhibition of toxin induced leak-

[7] This possibility is strengthened by the observation that although BSTP does not inhibit significantly when CB-dextran efflux is initiated at the time toxin is added to the vesicles and BSTP (Fig. 2), it inhibits CB-dextran influx strongly in samples with trapped anti-CB. In these samples toxin and BSTP were preincubated prior to the initiation of influx by addition of CB-dextran (*data not shown*).

age by Cd^{2+} indicates some effective inhibitors could have very different structures than the molecules tested here [1].

Applications of Inhibitors and Future Studies

Whatever the mechanism of inhibition, further studies of the effect of inhibitors on pore formation may yield information on the role of pore formation in the mechanism by which the A chain of the toxin translocates across membranes. In this regard, it is interesting that it has been observed that Cibacron Blue blocks translocation of the catalytic A chain of the toxin into the cytoplasm of cultured Vero cells [9]. This inhibition was proposed to be an indirect effect resulting from the effect of Cibacron Blue on cellular ion transport. However, in view of our results it is possible direct interaction with diphtheria toxin is involved. Therefore, it would be interesting to examine whether inhibitors can block toxin translocation in an in vitro system, where no other cellular proteins are present [12].

Finally, additional studies on inhibitors may also yield clues to therapeutically useful agents that block the action of diphtheria toxin and other protein toxins on cells. This would be of particular significance if inhibitors can be identified for toxins in which pore formation is the critical toxin-induced lesion, such as the α-hemolysin of *Staphylococcus aureus* [10, 30].

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