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STRUCTURAL FEATURES OF THE BOTULINUM NEUROTOXIN MOLECULE THAT GOVERN BINDING AND TRANSCYTOSIS ACROSS POLARIZED HUMAN INTESTINAL EPITHELIAL CELLS*

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Abbreviations: BoNT, botulinum neurotoxin; DTT, dithiothreitol; HA, hemagglutinin; HC-100, 100 kDa toxin heavy chain; HC-50, 50 kDa carboxy-terminal heavy chain fragment; MED, minimum essential domain; NTNH, non-toxin non-hemagglutinin; SAR, structure-activity relationships; TEER, transepithelial electrical resistance.

ABSTRACT

Experiments were done to help localize the minimum essential domain within the botulinum toxin molecule that is necessary for binding and transport across human gut epithelial cells. The data demonstrated that the neurotoxin alone, in the absence of auxiliary proteins, undergoes transcytosis. The neurotoxin by itself was examined in the single chain (unnicked serotype B) and dichain (nicked serotype B, nicked serotype A) forms, and all displayed the ability to bind and penetrate epithelial barriers. In addition, the single chain and dichain molecules were examined in the oxidized and reduced states, and again all forms were transported. To further define the minimum essential domain, experiments were done with two toxin fragments: 1) the heavy chain, which was derived from native toxin, and 2) the carboxy-terminal portion of the heavy chain, which was generated by recombinant techniques. Interestingly, both fragments were fully competent in crossing epithelial barriers. These data suggest that a polypeptide derived from the toxin could be used as a carrier domain to transport other molecules across epithelial cells. In related experiments, physiologic (i.e., potassium depletion) and pharmacologic (i.e., chlorpromazine) manipulations were used to implicate clathrin-coated pits/vesicles as the structures responsible for endocytosis of toxin.

INTRODUCTION

Botulinum neurotoxin (BoNT) is synthesized and released by *Clostridium botulinum* as seven serologically distinct proteins designated A through G (Oguma et al., 1995; Simpson, 2004). Some of these serotypes are released as single chain molecules that need further processing, enzymatic "nicking", to become dichain molecules. The remaining serotypes are "nicked" and released by clostridia as mature dichain neurotoxins (Oguma et al., 1995; Simpson, 2004). The dichain molecule includes a heavy chain (~100 kDa, HC-100) and a light chain (~50 kDa) linked by a disulfide bond. These mature neurotoxin molecules are ordinarily part of a complex formed by non-covalent association with other proteins, including a family of hemagglutinins (HA) and a single non-toxin non-hemagglutinin (NTNH) subunit (Oguma et al., 1995; Inoue et al., 1996; Herreros et al., 1999; Humeau et al., 2000).

BoNT is the etiologic agent responsible for the disease botulism, which is characterized by peripheral neuromuscular blockade (Simpson, 2004). In order to cause botulism the neurotoxin has to enter the body and be absorbed into the circulation. Most cases of poisoning are caused by ingestion of toxin, so it is clear that BoNT must escape the gastrointestinal system and reach the general circulation (lymph and blood) in route to peripheral cholinergic nerve endings. Earlier studies have identified the upper small intestine as the primary site of toxin absorption (Dack, 1926; Coleman, 1954; May and Whaler, 1958; Heckly et al., 1960).

There is an emerging body of research on the cellular and sub-cellular events that enable the toxin to cross membranes in the gastrointestinal system. Thus, our laboratory has attempted to delineate a mechanism for toxin transport across gut cells that involves binding to receptors on the mucosal side of the intestinal epithelium and transcytosis across cells (Maksymowych and Simpson, 1998; Maksymowych et al., 1999; Park and Simpson, 2003). *In vivo* experiments

demonstrated that neurotoxin, when placed directly into the upper intestine, escaped the lumen of the gut to reach the general circulation and eventually the neuromuscular junction (Maksymowych et al., 1999). *In vitro* experiments demonstrated that neurotoxin specifically bound to intestinal epithelial cells, was internalized, transported, and delivered intact and unmodified to the opposite side of the polarized epithelium. Furthermore, transcytosed material was fully active, as indicated by its ability to cause neuromuscular blockade when added to phrenic nerve-hemidiaphragm preparations or when injected into mice (Maksymowych and Simpson, 1998). This same sequence of events has been demonstrated for toxin transcytosis in lung epithelial cells (Park and Simpson, 2003), suggesting that toxin employs a common mechanism for penetration of epithelial barriers.

Although significant progress is being made in terms of identifying the steps that govern toxin binding and transcytosis across gut and airway epithelial cells, there still is much that remains to be learned. One area in which the deficiencies in our understanding are especially great is that of structure-activity relationships. For example, it is clear that the entire toxin molecule is necessary to produce neuromuscular blockade, but it is not known which portion of the holotoxin is essential for binding and penetration of epithelial barriers. As another example, it has been established that the auxiliary proteins HA and NTNH help protect the toxin against the harsh conditions of low pH and proteolytic enzymes in the gut, but it has not been determined whether these proteins hinder or facilitate the process of binding and transcytosis.

In the work that follows, an effort has been made to clarify the structure-activity relationships that are crucial to the ability of BoNT to be absorbed across the gut epithelium. This work is intended to achieve two broad goals: 1.) identify the minimum essential domain within the BoNT molecule that is necessary and sufficient for binding and transcytosis, and 2.)

establish the role, if any, of HA and NTNH in the absorption of BoNT.

METHODS

Materials - T-84 human colon carcinoma cells and Madin-Darby canine kidney cells were obtained from the American Type Culture Collection (Rockville, MD). Tissue culture media and sera were purchased from GIBCO™, Invitrogen Corporation (Carlsbad, California). [^{3}H]-inulin and [^{125}I]-Bolton-Hunter reagent were purchased from NEN (Boston, MA). Reagents were purchased from SIGMA Chemical Company (St. Louis, MO), and tissue culture supplies were obtained from Fisher Scientific (Malvern, PA). DTT was purchased from Roche Applied Science (Indianapolis, IN), and Amicon Centricon® centrifugal filters (YM-10 and YM-30) were obtained from Millipore Corp. (Bedford, MA). Rabbit polyclonal sera against BoNT/A and mouse polyclonal sera against BoNT/B were produced in our laboratory. Secondary antibodies, anti-mouse IgG HRP-linked whole antibody (sheep), anti-rabbit IgG HRP-linked whole antibody (donkey), and Sephadex G-25 gel filtration columns were obtained from Amersham Pharmacia Biotechnology Inc. (Piscataway, NJ).

BoNT/A and BoNT/B were purified according to procedures described in the literature (Sakaguchi, 1982; DasGupta and Sathyamoorthy, 1984; Simpson et al., 1988). Isolation and purification of serotype A HC-100 was also performed as previously described (Sathyamoorthy and DasGupta, 1985).

Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA). A Rapid DNA Ligation kit and an Expand High Fidelity PCR kit were purchased from Roche Applied Science (Indianapolis, IN). The expression vector pQE-30 and Ni-NTA Agarose were purchased from QIAGEN (Chatsworth, CA). Monoclonal antibodies specific for the His_6 affinity tag were purchased from QIAGEN. Plasmid pGEX.BoNT/A-HC carrying BoNT/A HC-100 DNA was kindly provided by Dr. Nikita

Kiyatkin.

Expression and purification of recombinant 50,000 dalton carboxy-terminal fragment of heavy chain. - Cultures were grown in Lennox L broth at 37°C, with shaking, to an A_{600} of 0.6 - 0.8. Isopropyl-β-D-thiogalactopyranoside was added to 1.0 mM final concentration, and incubation continued for an additional 5 h at 32°C. Bacteria from 1 liter of induced culture were harvested by centrifugation at 4°C and resuspended in 20 ml of 50 mM sodium phosphate buffer, pH 7.4, with 300 mM NaCl. The cell suspension was lysed, on ice, by sonication, with 2 pulses of 1 min duration each at 75% power, using a Model 60 Sonic Dismembrator (Fisher Scientific, Malvern, PA). Lysates were centrifuged at 20,000 x g for 30 min at 4°C. The clarified supernatants were mixed with 1 ml of packed Ni-NTA resin, incubated for 1h at 4°C on a rotator and finally poured into a 25 ml column. The column was washed with 50 volumes of washing buffer (50 mM sodium phosphate, pH 6.0, 300 mM NaCl, 25 mM imidazole). Bound proteins were eluted with elution buffer (50 mM sodium phosphate, pH 4.5, 300 mM NaCl). Purified proteins were analyzed on sodium dodecylsulfate polyacrylamide gels (SDS-PAGE).

Recombinant proteins, for analysis by Western blotting (see below), were separated on 10% polyacrylamide gels according to Laemmli (Laemmli, 1970), transferred to nitrocellulose, and processed for detection of immunoreactive proteins.

Iodination - The holotoxins of BoNT/A and BoNT/B, as well as HC-100 and HC-50, were iodinated using [^{125}I]-Bolton Hunter reagent essentially according to manufacturers instructions. The reaction time was reduced to diminish the loss of toxicity of the resulting product. The toxins were labeled to an average specific activity of 500 Ci/mmol with a residual toxicity of greater than 90% (Simpson et al., 1993; Simpson et al., 1994; Coffield et al., 1997). Pure neurotoxin or fragment (300 µg) in borate buffer (pH 7.8; 200 µl) was added to dried,

iodinated ester and reacted on ice for 15 min. The reaction was terminated by addition of 50 µl of 1 M glycine in borate buffer for 15 min. The total reaction mixture (250 µl) and rinse (250 µl) were loaded onto a Sepahadex G-25 column that was pre-equilibrated with filtration buffer (150 mM Na_2HPO_4, 150 mM NaCl, 0.1% gelatin, pH 7.4). The labeled toxin was eluted with filtration buffer, and 0.5 ml fractions were collected. An aliquot (5 µl) of each fraction was assayed for radioactivity. The labeled toxin peak, which eluted at void volume, was pooled and stored at 3°C. Toxin concentration in the pooled fraction was determined spectrophotometrically at 278 nm using the following relationship 1.63 A_{278} = 1 mg/ml (DasGupta and Sathyamoorthy, 1984); HC-100 concentration was determined using the relationship 0.8 (A_{235} - A_{280}) / 2.51 = 1 mg/ml; and H_C concentration was determined using 1.70 A_{278} = 1 mg/ml. For dosage calculations involving complexes, the neurotoxin content was estimated to be 35% of the total protein in the complex with hemagglutinins (150 kDa/400 kDa) (Oguma et al., 1995; Inoue et al., 1996).

Iodinated samples were assayed in a γ-counter to ascertain the number of counts (dpm) present. Sample concentration and associated counts were used to calculate specific activity.

Tryptic Nicking of BoNT/B - In order to generate the dichain form of BoNT/B, the single chain toxin was proteolytically nicked with trypsin. To facilitate subsequent separation of toxin from the nicking enzyme, TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone) treated trypsin crosslinked to 4% beaded agarose was used (Immobilized Trypsin; PIERCE, Rockford, IL). The trypsin slurry was washed 3X with reaction buffer (10 mM Sodium Phosphate Buffer, pH 7.5). Toxin was added and incubated with enzyme at room temperature (23°C) for 1 hour at a 1:10 ratio of trypsin to toxin. After incubation, nicked toxin was separated from the beaded trypsin by filtration through a 0.2 µ centrifugal filter (Schleicher & Schuell

Centrex Microfilter Unit) into a sterile tube. The nicked toxin was collected, aliquotted and stored at -20°C until use. A sample of the material was examined by electrophoresis to verify degree of nicking and integrity of the dichain toxin.

Toxin reduction - BoNT/A or BoNT/B (both nicked and "un-nicked") were reduced by incubation with DTT for 1 h at room temp. The appropriate amount of a 40 mM stock solution of DTT was added to an aliquot of iodinated toxin to give a final concentration of 5 mM. After reduction the toxin aliquot was diluted into 3 ml of medium and subsequently used for transcytosis assay. During transcytosis experiments the final concentration of DTT in the upper Transwell® chamber was ≤ 0.085 mM.

Cell Culture - T-84 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (1 g/L D-glucose) and Ham's F-12 nutrient medium supplemented with 5% newborn calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 8 µg/ml ampicillin and 15 mM Hepes. Cultures were maintained at 37°C in 6% CO_2. T-84 cells were fed every three days and passaged (1:2) when 95% confluent, approximately every six days. Passages 65 through 90 were used for experiments described in this paper.

MDCK cells were grown in Dulbecco's modified Eagle's medium (4.5 g/L D-glucose) supplemented with 10% newborn calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 mM Hepes. Cultures were maintained at 37°C in 6% CO_2. MDCK cells were fed every three days and passaged (1:5) when 95% confluent, approximately every three days. Passages 56 through 85 were used for experiments described in this paper.

MDCK cells were used throughout this study as controls. MDCK cells differentiate into an epithelial membrane with tight junctions, but do not possess receptors for BoNT and therefore, do not transcytose BoNT.

Transcytosis Assay - For assay of iodinated molecule transcytosis, cells were grown in Transwell® porous bottom dishes on polycarbonate membranes with a 0.4 µm pore size. The growth area within each insert was 1 cm^2. Prior to being seeded with cells, the transwells were coated with 10 µg/cm^2 rat tail collagen type I. Briefly, rat tail collagen stock solution (6.7 mg/ml) was prepared in sterile 1% acetic acid and stored at 3°C. The stock solution was diluted in acidified 60% ethyl alcohol, as needed and 150 µl containing 10 µg of diluted collagen was added to each well. The coated wells were allowed to dry at room temperature overnight (18 h). After drying, the wells were sterilized under UV light for one h, followed by preincubation with cell culture medium (30 min). The preincubation medium was removed immediately prior to addition of cells and fresh medium.

Cells (T-84 or MDCK) were plated at confluent density (ca. 1.5 x 10^5 cells) into the transwells with 0.5 ml medium in the upper chamber and 1.0 ml in the lower chamber. In these experiments, medium in the upper chamber bathed the apical (or mucosal) surface of cells, and medium in the lower chamber bathed the basolateral (or serosal) surface of cells. Culture medium was changed every two days. The cultures were allowed to differentiate for a minimum of 10 days before assay of transcytosis. The formation of tight junctions was experimentally confirmed by measuring the rate of [^{3}H]-inulin movement from the upper chamber into the lower chamber (see below). Experiments were performed on cultures that were between 10 and 15 days old.

The transcytosis assay was initiated by adding 1 x 10^{-8} M [^{125}I]-labeled botulinum neurotoxin or fragment to the upper (A→B) or bottom chamber (B→A). Transport of radiolabeled molecules was monitored by collecting all of the medium from the appropriate chamber. An aliquot (0.5 ml) from each sample was filtered through a Sephadex G-25 column,

and 0.5 ml fractions were collected. The amount of radioactivity in the fractions was determined in a γ-counter. Labeled toxin or fragments eluted at void volume, and the radioactivity contained in the void volume fractions was summed to determine the total amount of labeled protein present. Transcytosis was expressed as fmol/cm^2/hr.

Samples of transcytosed toxin or toxin fragments for Western blots were obtained by performing transcytosis experiments in medium with 0.2 % serum in the top chamber of the transwell and PBS in the bottom well. At an appropriate time point, the entire contents of three wells per condition (3 ml) were collected and concentrated in Centricon™ microconcentrators. Centricon™ 30's were used for molecules of 100 kDa and larger whereas centricon 10's were used for concentration of molecules below 100 kDa. Collected samples were concentrated to a final volume of 40 µl, of which 10 µl/lane were loaded onto gels.

Membrane integrity - To control for membrane integrity and to estimate diffusion between cells, the rate of passage of [^{3}H]-inulin from the upper to the lower reservoir was determined as described previously (Madara and Dharmsathaphorn, 1985).

In addition to inulin transport, transepithelial electrical resistance (TEER) was used to evaluate the structural integrity of T-84 and MDCK epithelial cells for transcytosis experiments. An epithelial volt-ohmmeter (EVOM, World Precision Instruments, Sarasota, FL) was used to monitor electrical resistance across the cell monolayers during experiments. TEER for cultured membranes included in experiments was ≥ 400 Ω cm^2 for T-84 cells and ≥ 600 Ω cm^2 for MDCK cells. Controls were empty wells with collagen-coated polycarbonate membranes immersed in assay medium. Resistance was calculated as Ohms per square centimeter.

As an additional control for membrane integrity, each transwell was checked for leakage at the end of each experiment. For this purpose, medium was added to the upper chamber but

not the lower chamber. The Transwell® apparatus was returned to the incubator at 37°C, and evidence of fluid accumulation in the lower reservoir was monitored for six hours. Leakage seldom occurred (< 3 percent of wells); when there was leakage, the data from these wells were not included in the final analysis.

Western blot analysis. - Rabbit immune serum against BoNT/A toxin was generated in our laboratory. Samples for Western blot analysis were separated in 10 % SDS-polyacrylamide gels according to Laemmli (Laemmli, 1970). Subsequent to separation, proteins were transferred to NitroPure membranes (Micron Separations Inc., Westboro, MA) in Tris-glycine transfer buffer at 50 volts for 1 hour. Blotted membranes were rinsed in distilled water and stained for 1 minute with 0.2% Ponceau S in 1% acetic acid. Following a brief rinse with distilled water, molecular weight markers and transferred proteins were identified. Membranes were destained in phosphate buffered saline-Tween (pH 7.5; 0.1% Tween 20), blocked with 5% non-fat powdered milk in phosphate buffered saline-Tween overnight at 3ºC. For identification of toxin or recombinant fragments, membranes were washed again (3X) and incubated with primary immune serum at 1:5,000 dilution, in 0.05 to 1.0% milk overnight at 3°C. Membranes were washed (3X) and incubated with secondary antibody at 1:20,000 dilution for 1 h at room temperature.

Membranes were washed again (3X) and visualized using enhanced chemiluminescence (ECL; Amersham-Pharmacia Biotech, Piscataway, NJ.) according to manufacturer's instructions. Membranes were exposed to film (Hyperfilm-ECL) for times adequate to visualize chemiluminescent bands. Peptides were identified by comparison with known standards.

Potassium depletion experiments - For assay of transcytosis in potassium depleted cultures, polarized cells in transwells were pre-incubated for 30 min at 37°C in potassium-

depletion buffer: 20 mM HEPES, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 4.5 mg/liter glucose, pH 7.4. After preincubation, fresh potassium depletion buffer was added to both top and bottom wells and transcytosis of toxin was assayed for 3 hours, as described above.

Chlorpromazine treatment - For assay of transcytosis by chlorpromazine pretreated cultures, polarized cells in transwells were exposed to medium containing 25 µg/ml chlorpromazine in both top and bottom wells for 1 h at 37°C. After preincubation, fresh chlorpromazine-containing medium was added to both top and bottom wells and transcytosis of toxin was assayed for 3 hours, as described above.

RESULTS

Structure and processing of botulinum neurotoxin – For the purposes of this study, botulinum toxin can be envisioned as having four molecular states (Fig. 1): 1) single chain toxin with an intact intrachain disulfide bond, 2) single chain toxin with a reduced intrachain disulfide bond, 3) dichain toxin with an intact interchain disulfide bond, and 4) dichain toxin with a reduced interchain disulfide bond.

All serotypes of botulinum toxin are synthesized as single chain molecules. Some organisms, such as those that manufacture serotype B, may release the toxin without further processing, whereas other organisms, such as those that synthesize serotype A, typically process the toxin (i.e. nicking). Therefore, the study has utilized BoNT/B as an example of single chain toxin and BoNT/A as an example of dichain toxin.

The single chain and dichain molecules found in culture media normally have an intact intrachain/interchain disulfide bond. This disulfide can be reduced experimentally (see Methods) but it is also reduced in the interior of target cells. Thus, there is evidence that nerve terminals that endocytose the toxin subsequently reduce the disulfide during or immediately after translocation of the toxin to the cytosol. Therefore, experiments were done on the single chain and dichain species in both the oxidized and reduced forms.

Previous work on isolated neuromuscular preparations, as well as other neuronal or synaptosomal preparations, suggests that the HC-100 plays a role of fundamental importance in binding to cell surface receptors. Indeed, the binding domain may be localized to the carboxy-terminal half of the HC-100. Therefore, studies on structure-activity relationships included not only the four molecular states of the holotoxin, but also the isolated HC-100 (derived from native toxin) and the isolated carboxy-terminal half of the HC-100 (expression product).

Finally, it must be noted that BoNT in culture media rarely exists as the isolated protein. More typically, the toxin is found in a non-covalent complex with HA and NTNH. This complex likely remains intact during toxin transit through the gut (viz., protects toxin from metabolic degradation; see Introduction), but there is no evidence that the complex remains intact within the general circulation. More likely, the complex dissociates and then free toxin reaches and paralyzes cholinergic cells. Given these facts, the present study has made a qualitative assessment of whether HA and NTNH are essential for single chain or dichain toxins (both in the oxidized form) to cross epithelial barriers.

Transcytosis of neurotoxin complex vs. pure neurotoxin – Qualitative experiments were done to determine whether HA and/or NTNH is essential for BoNT to bind and penetrate epithelial cells. For this purpose, equimolar concentrations of neurotoxin in complex and neurotoxin free of auxiliary proteins were assayed for their respective abilities to cross gut epithelial cells (T-84) and kidney epithelial cells (Fig. 2).

An analysis of the single chain specie (BoNT/B) in the oxidized form revealed that both the complex and the isolated toxin could cross gut epithelial barriers (Fig. 2, Panel A). However, neither the complex nor the isolated toxin was efficient in crossing kidney epithelial barriers. Analysis of complex and free dichain toxin (BoNT/A) in the oxidized condition produced an identical outcome (Fig 2, Panel B). Both forms of the toxin crossed gut epithelial barriers, but neither efficiently crossed kidney epithelial barriers.

These results support three conclusions. First, botulinum toxin can cross gut epithelial cells in either the single chain or dichain form. Second, botulinum toxin does not have an absolute requirement for either HA or NTNH to bind and penetrate gut cells. Third, although both single chain and dichain molecules can cross gut epithelial cells, and although the binding

and transcytosis can occur either in the absence or presence of auxiliary protein, none of these variants displayed significant ability to cross kidney epithelial cells.

Transcytosis of unnicked and nicked BoNT/B – One further experiment was done to ensure that transcytosis of the single chain and dichain species was comparable. Rather than comparing one serotype in the single chain state and a different serotype in the dichain state, a single serotype (BoNT/B) was examined in both states (viz., unnicked vs. nicked; see Methods for trypsin-induced nicking).

As shown in Table 1, the unnicked and nicked forms of BoNT/B crossed gut epithelial cells at similar rates. This was true, regardless of whether transcytosis was in the apical to basal direction or in the basal to apical direction. However, in keeping with earlier findings (Maksymowych and Simpson, 1998; Park and Simpson, 2003), it was observed that transcytosis of any particular specie of molecule (viz., single chain molecule) tends to be greater in the apical to basal direction than in the opposite direction.

The data in Table 1 may suggest that transcytosis of unnicked toxin is slightly greater than that of nicked toxin. However, some caution must be exercised in analyzing these data. Conversion from the unnicked to the nicked state requires exposure of the toxin to the endoproteolytic actions of trypsin (see Methods). For a small fraction of the toxin molecules, trypsin may have exerted an endoprotease effect beyond that of simple nicking, and this may have had a slight effect on overall rates of transcytosis.

Finally, the data with single chain and dichain forms of BoNT/B confirm data above. These molecules are able to cross gut epithelial barriers, but they are not as able to cross kidney epithelial barriers.

Transcytosis of oxidized vs. reduced states of the single chain and dichain molecules

– Quantitative experiments were done in which the apical to basal rate of transcytosis was measured for the four molecular states of the toxin (see above). For this purpose, unnicked BoNT/B was used as an example of a single chain toxin, and both nicked BoNT/B and nicked BoNT/A were used as representative dichain toxins. The results of these experiments are presented in Table 1.

For unnicked BoNT/B, the rates of transcytosis of oxidized and reduced toxin across monolayers of T-84 cells were closely comparable (9.01 ± 0.44 fmol/cm^2/hr vs. 8.96 ± 0.80 fmol/cm^2/hr). A similar pattern was observed with the two dichain toxins. Thus, oxidized and reduced forms of nicked BoNT/B (6.57 ± 0.07 fmol/cm^2/hr vs. 5.17 ± 0.21 fmol/cm^2/hr) and of nicked BoNT/A (12.61 ± 0.28 fmol/cm^2/hr vs. 11.94 ± 0.21 fmol/cm^2/hr) were quite similar.

In contrast to the data on gut epithelial cells, the data on kidney epithelial cells revealed rates of transcytosis that were too low to permit meaningful comparisons between oxidized and reduced molecular states.

***In vitro* toxicity of oxidized, nicked BoNT/B after transcytosis** - The dichain form of botulinum toxin with an oxidized disulfide bond is the active state that accounts for neuromuscular blockade. Previous work has demonstrated that, when this form of BoNT/A is transcytosed by T-84 cells, it is released in a form that is structurally intact and biologically active (Maksymowych and Simpson, 1998). Thus, transcytosed material retains its immunologic characteristics, as revealed in Western blots, and it retains toxicity, as measured on isolated neuromuscular preparations.

To ensure that the dichain oxidized form of BoNT/B retains its distinctive characteristics after transcytosis, experiments were done with polarized monolayers of T-84 cells and isolated phrenic nerve-hemidiaphragm preparations. The active state of the toxin was added to the apical

surface of several monolayers. Aliquots of material from the basal surface were collected to achieve a final concentration of 10^{-11} M neurotoxin (calculated on the basis of rate of transcytosis; see above). This material was added to phrenic nerve-hemidiaphragm preparations, and the time-to-paralysis for these tissues was compared to that of tissues that were treated with native toxin (i.e., not submitted to binding and transcytosis). The resulting paralysis times were strikingly similar (131 ± 14 min for transcytosed toxin; 139 ± 17 for native toxin). These findings indicate not only that gut epithelial cells can bind and transcytose BoNT/B, but also that the material released from cells retains full biological activity.

Transcytosis of BoNT/A HC-100 and recombinant HC-50 – Studies of BoNT binding to nerve terminals have demonstrated that the receptor-binding region is localized to the carboxy-terminal portion of the HC-100 (Simpson et al., 1999; Schiavo et al., 2000; Simpson, 2004). The work described in the preceding sections has shown that various molecular states of BoNT can bind and penetrate epithelial barriers. A logical extension of this work is to determine the location of the receptor-binding domain that accounts for interaction with epithelial cells.

As before, experiments were done with polarized monolayers of T-84 cells and polarized monolayers of MDCK cells. An initial series of qualitative experiments were done to determine whether either the native HC-100 or the recombinant HC-50 would bind and be transported. As shown in Figure 3, immunodetection experiments with immunoblots demonstrated that holotoxin, HC-100, and HC-50 were all capable of binding and transcytosis. As would be predicted, none of the three molecules effectively crossed MDCK cells.

In the next set of experiments, the individual polypeptides were iodinated, and the actual rates of transcytosis were measured. As detailed in Figure 3, the rates of transcytosis for HC-100 (7.10 ± 0.00 fmol/cm^2/hr) and for HC-50 (14.46 ± 0.01 fmol/cm^2/hr) were in the same range as

that for holotoxin (11.29 ± 0.30 fmol/cm^2/hr). These data support the conclusion that the receptor-binding domain for interaction with epithelial cells is localized somewhere within the 50,000 dalton polypeptide (HC-50) that represents the C-terminal portion of the holotoxin.

Effects of potassium depletion on transcytosis of BoNT/A – It is important to identify that portion of the toxin molecule that interacts with the cell, as indicated by the previous series of studies. It is equally important to identify that portion of the cell that interacts with the toxin. Previous work (Maksymowych and Simpson, 1998; Maksymowych et al., 1999; Park and Simpson, 2003), as well as the data presented above, suggest that BoNT crosses epithelial barriers by the process of receptor-mediated endocytosis, transcytosis, and subsequent release. A cell structure that is a candidate to participate in this process is the clathrin-coated pit/vesicle.

Earlier studies on a variety of cell types have shown that potassium depletion disrupts the formation of clathrin-coated pits and significantly inhibits clathrin-mediated endocytosis (Subtil et al., 1994; Minana et al., 2001; Wu et al., 2001; Huang et al., 2002). Therefore, experiments were done with epithelial monolayers that had been preincubated in potassium depletion buffer (see Methods). The data, which are presented in Figure 4, clearly show that binding and transcytosis of iodinated BoNT/A is significantly reduced in T-84 cells in which the clathrin-mediated pathway has been disrupted. Potassium depletion buffer led to a 68% inhibition of transcytosis.

To guard against the possibility that potassium depletion buffer could have some non-specific effect that disrupted cell structure or function, an additional series of experiments was performed. TEER was measured over time in control cells and in experimental cells. The data (Fig. 4) indicated that cells bathed in control medium and cells pretreated with potassium depletion buffer were closely comparable.

Effects of chlorpromazine on transcytosis of BoNT/A – As a further test of the putative involvement of clathrin-mediated endocytosis, experiments were done with a pharmacologic agent that disrupts the clathrin pathway by a mechanism different from that of potassium depletion. The drug chlorpromazine induces redistribution of AP-2, a component of clathrin-coated pits, and has been shown to inhibit internalization of transferrin, a marker for the coated pit pathway (Subtil et al., 1994; Okamoto et al., 2000; Shogomori and Futerman, 2001; Huang et al., 2002).

Transcytosis of iodinated BoNT/A was measured in T-84 cell monolayers in the presence of 25 µg/ml chlorpromazine (Fig. 5). This drug had a striking effect, inhibiting transcytosis by approximately 80 to 90%. Similarly to the studies with potassium depletion buffer, the studies with chlorpromazine showed that transcytosis could be markedly inhibited without disrupting TEER.

DISCUSSION

There is a significant body of information on the mechanisms by which botulinum toxin interacts with epithelial cells (viz., transport cells) and cholinergic nerve cells (viz., target cells). This information reveals that there are both major similarities and major dissimilarities in the two phenomena. Both processes begin with toxin binding to a cell surface receptor. Interestingly, toxin association with these receptors does not produce any adverse effects on cell function.

At both transport cells and target cells, receptor binding leads to receptor-mediated endocytosis that is followed by a cascade of receptor-dependent events. However, a comparison of transport cells and target cells reveals that there are substantial differences in the nature of the endocytic vesicle and in the fate of internalized toxin. In polarized epithelial cells, internalized toxin is directed to a population of endocytic vesicles that mediate transcellular transport. These vesicles cross the cell body, meld with the basolateral membrane, and discharge toxin into the extracellular space. Importantly, the toxin is not modified during transport; instead it is released on the basolateral surface as an intact dichain molecule that can paralyze cholinergic transmission (Maksymowych and Simpson, 1998; Maksymowych et al., 1999; Park and Simpson, 2003).

Toxin that enters nerve endings is largely confined to early endosomes that possess a membrane proton pump. Acidification of the lumen causes marked structural changes in the toxin molecule. Although many details of the process are still unknown, one can deduce that the toxin inserts into the endosome membrane, and this is accompanied by reduction of the disulfide bond, separation of the two chains, and eventual escape of the light chain to the cytosol. Productively internalized toxin that reaches the cytosol blocks exocytosis (Simpson, 2004).

Although toxin interaction with transport cells and toxin interaction with target cells both

begin with a receptor binding step, the level of understanding of these two events is sharply different. There is a significant amount of data that defines the structure-activity relationships (SAR) at nerve endings, but there are no such data for epithelial cells. At the nerve ending, the HC-100 is essential for binding and productive internalization, although the light chain may facilitate the process (Daniels-Holgate and Dolly, 1996). Within the HC-100, the HC-50 appears to possess the receptor binding domain (Lacy and Stevens, 1999; Lalli et al., 1999; Swaminathan and Eswaramoorthy, 2000). There is an emerging literature seeking to define the specific amino acids and the 3-dimensional characteristics of HC-50 that mediate binding (Lacy et al., 1998; Lacy and Stevens, 1999; Lalli et al., 1999; Ginalski et al., 2000; Hanson and Stevens, 2000; Swaminathan and Eswaramoorthy, 2000).

In contrast to the situation with nerve endings, there is no SAR literature that defines toxin interaction with epithelial cells. Therefore, the present study was intended to begin the characterization of toxin binding to epithelial cells. The goal was to address four specific issues: 1) the role of auxiliary proteins in binding and transcytosis, 2) the role of nicking and of the interchain/intrachain disulfide bond in binding and transcytosis, 3) localization of the minimum essential domain (MED) within the toxin that is essential for toxin association with cells, and 4) identification of the vesicle population that is linked to receptor binding.

Role of auxiliary proteins. Data presented here (Fig. 2) and elsewhere (Maksymowych and Simpson, 1998; Maksymowych et al., 1999; Park and Simpson, 2003) show that auxiliary proteins are not required for the neurotoxin to cross epithelial barriers. For example, homogeneous neurotoxin administered directly into the upper small intestine, which is the principal site of toxin absorption, crosses epithelial cells to reach the general circulation and eventually the neuromuscular junction (Maksymowych et al., 1999). This is in keeping with the

data in Figure 2. BoNT/A and BoNT/B, when added to the apical surface of human gut epithelial cells, underwent binding and transcytosis. This phenomenon did not depend on the presence of HA or NTNH.

The data on *in vivo* and *in vitro* transport are, in part, supported by findings on gene structure. Although most strains of clostridia that possess a gene for toxin also possess a gene for hemagglutinin, there are many strains that do not. Thus, investigators have identified strains of BoNT/A, BoNT/E, and BoNT/F that are devoid of the message encoding hemagglutinin (Sakaguchi et al., 1990; East et al., 1996; Johnson and Bradshaw, 2001). Clearly, the neurotoxin from these organisms cannot depend on the presence of hemagglutinin for absorption from the gut.

The role of nicking and the disulfide bond. As shown in Figure 1, the neurotoxin molecule can exist in four states. The neurotoxin is manufactured as a single chain polypeptide, but this molecule must undergo post-translational nicking to become fully active. Similarly, the toxin is made with an intact disulfide bond, but this bond must be reduced for the toxin to express its intraneuronal catalytic activity.

One of the most striking observations to emerge from this study is that toxin interaction with epithelial cells is less selective than toxin interaction with neuronal cells. In the former, there is no obvious discrimination among the four states of the toxin. Binding and transcytosis occur, regardless of nicking or disulfide bond reduction.

There are two implications that arise from the observation that epithelial cells transport all four states of the toxin. The first of these relates to the molecule that has undergone nicking and disulfide bond reduction. The fact that the holotoxin is delivered to the basolateral side of epithelial cells means that the non-covalent forces between the heavy and light chains are

sufficient to keep the molecule "intact" during transport.

The second implication of the data relates to SAR analyses. It is clear that the limited proteolysis associated with nicking, as well as the reduction of the interchain disulfide bond, does not produce conformational changes that alter the binding properties of the toxin. By extension, one might hypothesize that the structural elements of the toxin molecule that are involved in binding are independent of – and perhaps even remote from – the structural elements involved in nicking and maintenance of the disulfide bond.

Localization of the MED. The way in which botulinum toxin interacts with receptors and endocytic vesicles at the nerve ending is somewhat complex, although it does appear that the carboxy-terminal half of the HC-100 is essential for the process. Therefore, experiments were done to assess the ability of the isolated HC-100 (native protein) and the isolated HC-50 of this chain (recombinant protein) to cross epithelial barriers. Interestingly, both polypeptides were fully competent in achieving binding and transcytosis. These results are somewhat different from those that other investigators have obtained with nerve cells. The identity of the minimum polypeptide needed for binding and productive internalization at nerve endings remains unclear (i.e., compare Daniels-Holgate and Dolly, 1996 with Lalli et al., 1999).

The finding that HC-100, and even HC-50, can bind and be endocytosed helps to clarify the structure-activity relationships that govern interactions with epithelial cells. These findings also help in the effort to compare events at transport cells and target cells. However, there may be another and less obvious implication of the data that could ultimately be far more important.

The fact that HC-50 is fully competent to be transported across epithelial cells supports a change in perspective and terminology. In functional terms, the 50 kDa polypeptide can be referred to as a "carrier domain", and the remainder of the toxin molecule can be referred to as

the "cargo domain". It is inherent in the data that the naturally occurring cargo domain can be removed, and the carrier domain retains full activity. This raises a provocative question: can an alternative cargo domain be added to the carrier, and will this chimeric molecule undergo binding and transcytosis?

In the recent past, the authors have constructed a large number of chimeric molecules in which the botulinum toxin carrier domain was attached to heterologous cargo molecules (Simpson et al., Patent pending). These chimeric molecules covered a wide range of molecular weights and functional characteristics, and in every case the novel constructs were shown to cross epithelial barriers (Maksymowych et al., manuscript in preparation). These results support the premise that the botulinum carrier domain could be the foundation for creating an entirely new class of oral drugs.

Identification of the vesicle population. An initial attempt was made to gain insight into the nature of the vesicle population that mediates endocytosis and transcytosis of botulinum toxin. In preliminary experiments, a variety of agents that affect membrane cholesterol and lipid rafts were tested, but none had a specific effect on binding and transport of the toxin (Maksymowych and Simpson, unpublished data). On the other hand, two widely used procedures that typically affect clathrin-coated vesicles had a marked effect.

As shown in Figure 4, potassium depletion produced >50% inhibition in binding and transport of the toxin. An even greater effect (>80%) was achieved by pretreating cells in chlorpromazine. In both cases, measurements of transepithelial electrical resistance showed that the monolayers remained intact. These findings support the hypothesis that endocytosis of botulinum toxin into cells is mediated by clathrin-coated vesicles rather than lipid rafts.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Molecular states of botulinum neurotoxin that occur in nature or are generated in the laboratory. Botulinum neurotoxins occur in nature as either single chain or dichain molecules, each having either an oxidized or reduced intrachain/interchain disulfide bond. The four states are: **1.** Un-nicked neurotoxin with intact intrachain disulfide (e.g., Serotype B); **2.** Un-nicked neurotoxin with a reduced intrachain disulfide; **3.** Nicked neurotoxin with intact interchain disulfide (e.g., Serotype A); **4.** Nicked neurotoxin with a reduced interchain disulfide. The nicked and reduced form of the toxin can be separated into a light chain and a heavy chain (HC-100; **5**). The latter can be fractionated into an amino-terminal portion and a carboxy-terminal portion (HC-50; **6**) For the purposes of this study, molecules **1** to **5** were native proteins purified from clostridia, and molecule **6** was a recombinant protein obtained by expression in *E. coli*.

Figure 2. Transcytosis of BoNT/A and B complexes and pure neurotoxins in differentiated, polarized T-84 or MDCK cells. T-84 cells and MDCK cells were grown at 37°C on culture inserts, and transcytosis was assayed at 37°C as described under "Methods". A concentration of 1.0×10^{-8} M neurotoxin, either as complex or as homogeneous protein, was added to the top chamber of three transwells per cell line. After incubation at 37°C for 18 h, the media from individual basal wells were collected and concentrated using a Centricon™ concentrator. Ten µl of concentrate were loaded in each lane of a 10% polyacrylamide gel. After electrophoretic separation, gels were blotted onto nitrocellulose and probed with either rabbit anti-BoNT/A serum or with mouse anti-BoNT/B HC-100 serum. Western blots were visualized using chemiluminescent substrate. These blots are representative of 3 independent

experiments.

PANEL A: Lane 1, BoNT/B complex transcytosed in T-84; **Lane 2**, BoNT/B complex transcytosed in MDCK cells; **Lane 3**, Pure BoNT/B neurotoxin transcytosed in T-84 cells; **Lane 4**, Pure BoNT/B neurotoxin transcytosed in MDCK cells.

PANEL B: Lane 1, BoNT/A complex transcytosed in T-84 cells; **Lane 2**, BoNT/A complex transcytosed in MDCK cells; **Lane 3**, Pure BoNT/A neurotoxin transcytosed in T-84 cells; **Lane 4**, Pure BoNT/A neurotoxin transcytosed in MDCK cells.

Figure 3. Transcytosis of BoNT/A derived molecules in differentiated, polarized T-84 or MDCK cells. T-84 cells and MDCK cells were grown at 37°C on culture inserts, and transcytosis was assayed at 37°C as described under "Methods". **QUALITATIVE:** A polypeptide concentration of 1.0×10^{-8} M, as pure toxin, HC-100, or HC-50, was added to the top chamber of three transwells per cell line. After incubation at 37°C for 18 h, all media from the basal wells were collected and concentrated using a Centricon™ concentrator. Ten µl of concentrate were loaded in each lane of a 10% polyacrylamide gel. After electrophoretic separation, gels were blotted onto nitrocellulose and probed with anti-BoNT/A rabbit serum antibodies. Western blots were visualized using chemiluminescent substrate. This blot is representative of 3 independent experiments.

QUANTITATIVE: Rates of transcytosis were determined using [^{125}I]-protein. At the end of each experiment, the contents of the basal chamber were collected and assayed as described under "Methods". Rates were calculated based upon the specific activity of labeled toxin and duration of the experiment. The rates reported are mean ± sem at 37°C in the apical to basal direction.

Figure 4. Effect of potassium depletion on transcytosis of [^{125}I]-BoNT/A in polarized T-84 cells. Pure [^{125}I]-BoNT/A neurotoxin transcytosis was assayed in polarized T-84 cells for 3 hours. Experimental cultures were pre-incubated in potassium depletion medium for 1 hour prior to assay of transcytosis in depletion medium. Data are presented as average percent transytosis ± s.e.m. Each bar represents an average of 3 independent experiments for each condition assayed for transcytosis in the apical to basolateral direction. Light bar □, transcytosis of pure BoNT/A in control cultures. Dark bar ■, transcytosis of pure BoNT/A in potassium depletion medium. **Inset:** TEER for experimental cultures expressed as % control TEER value ± s.e.m, at various time points ●—●. Each point represents the average of 3 independent experiments.

Figure 5. Effect of chlorpromazine on transcytosis of [^{125}I]-BoNT/A in polarized T-84 cells. Pure [^{125}I]-BoNT/A neurotoxin transcytosis was assayed in polarized T-84 cells for 3 hours. Cultures were pre-incubated with or without 25 µg/ml chlorpromazine for 1 hour prior to assay of transcytosis. Data are presented as average percent transcytosis ± s.e.m. Each bar represents an average of 3 independent experiments for each condition assayed for transcytosis in the apical to basolateral direction. Light bar □, transcytosis of pure BoNT/A in control cultures. Dark bar ■, transcytosis of pure BoNT/A in chlorpromazine pretreated cultures. **Inset:** TEER for experimental cultures expressed as % control TEER value ± s.e.m, at various time points ●—●. Each point represents the average of 3 independent experiments.

Table 1. Transcytosis of Botulinum Neurotoxin

		Serotype B		Serotype A	
Single Chain (UN-NICKED)		**OXIDIZED**	**REDUCED**	**OXIDIZED**	**REDUCED**
APICAL ➡ BASAL					
	T-84	9.01 ± 0.44*	8.96 ± 0.80	—	—
	MDCK	0.05 ± 0.00	0.04 ± 0.00	—	—
BASAL ➡ APICAL					
	T-84	4.48 ± 0.40	N.D.	—	—
	MDCK	0.05 ± 0.00	N.D.	—	—
Dichain (NICKED)					
APICAL ➡ BASAL					
	T-84	6.57 ± 0.07	5.17 ± 0.21	12.61 ± 0.28	11.94 ± 0.21
	MDCK	0.05 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.00
BASAL ➡ APICAL					
	T-84	5.54 ± 0.08	N.D.	N.D.	N.D.
	MDCK	0.05 ± 0.00	N.D.	N.D.	N.D.

* Units of transcytosis are expressed as fmol/cm^2/hr.

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Figure 1

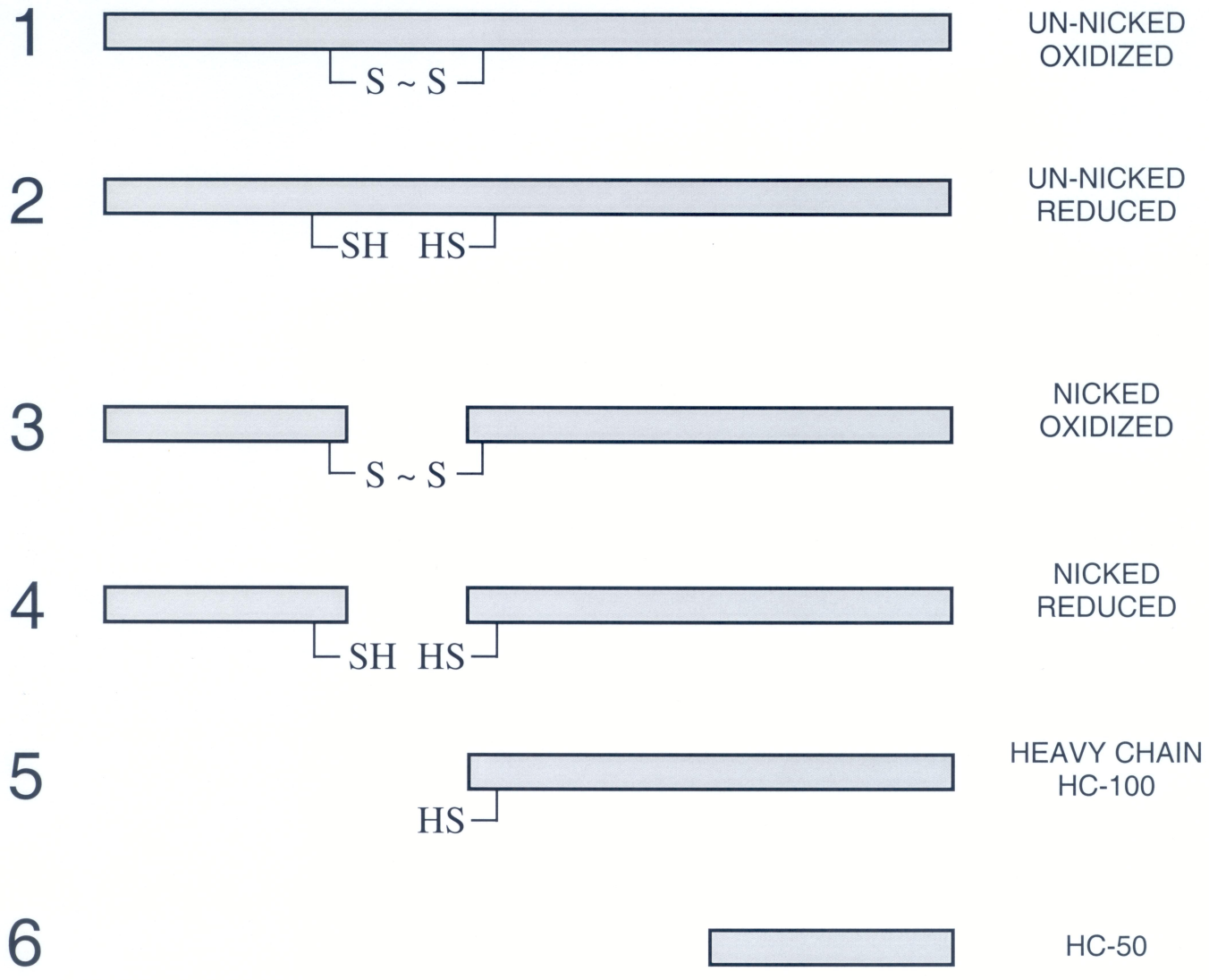

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Figure 2

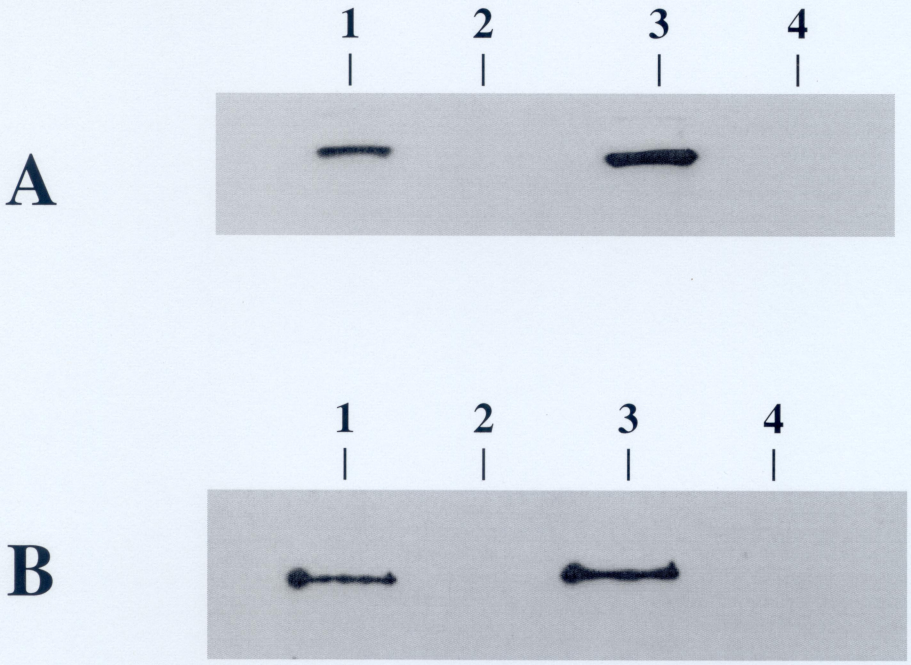

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Figure 3

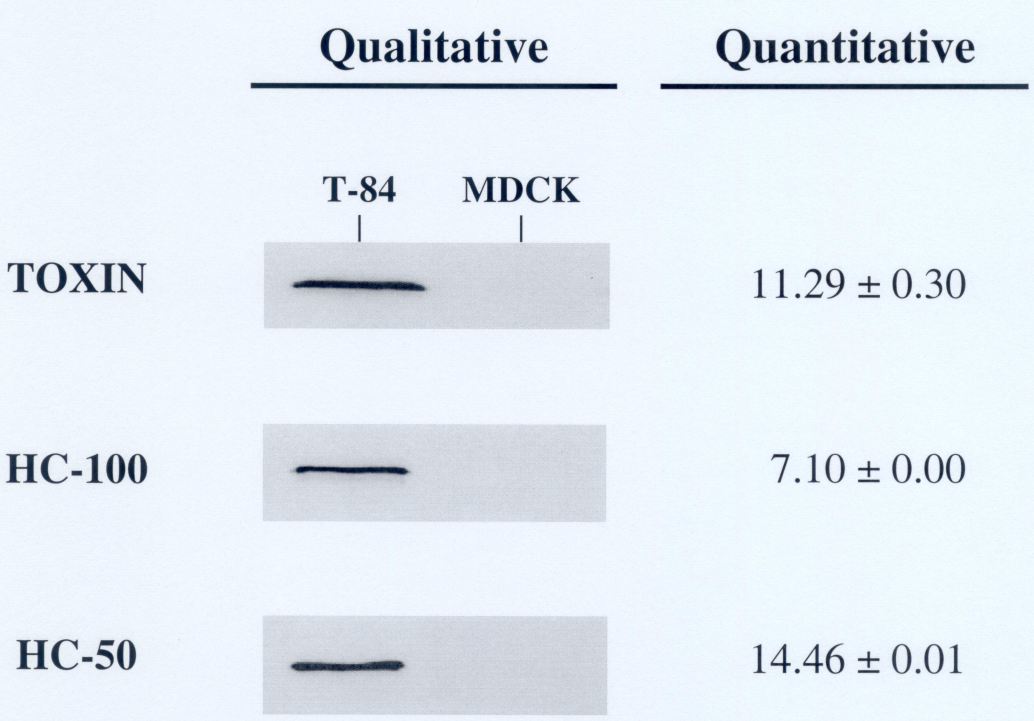

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Figure 4

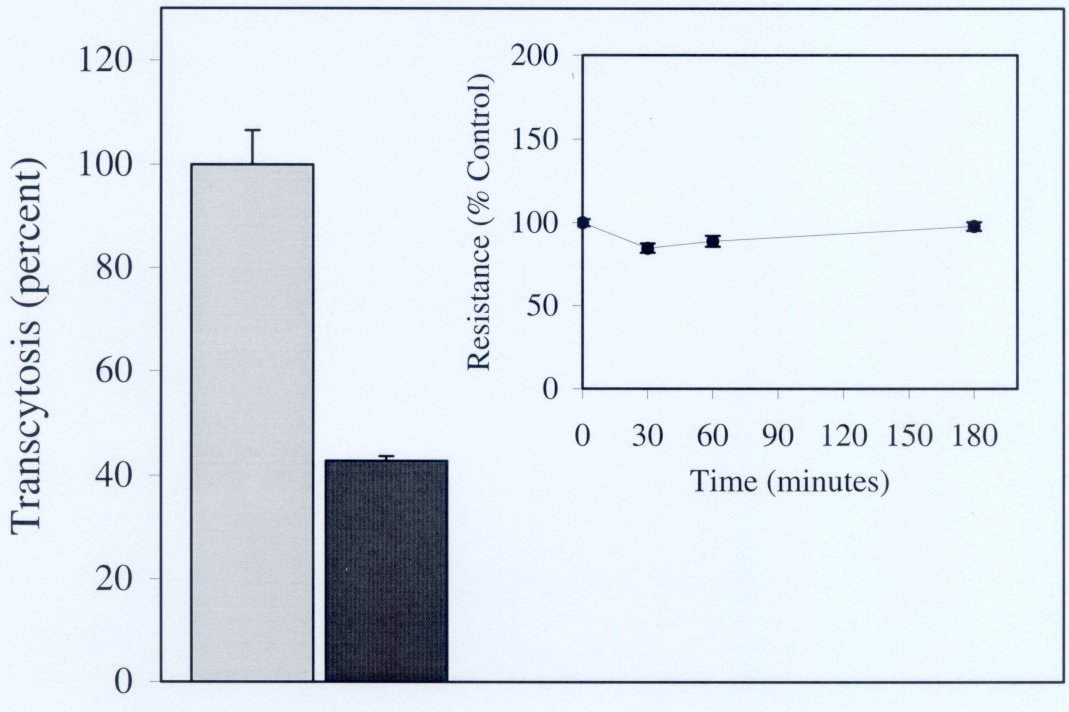

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Figure 5