The Cytopathic Activity Of Cholera Toxin Requires A Threshold Quantity Of Cytosolic Toxin.

2013

Carly Bader
University of Central Florida

THE CYTOPATHIC ACTIVITY OF CHOLERA TOXIN REQUIRES A THRESHOLD QUANTITY OF CYTOSOLIC TOXIN

by

CARLY BADER
B.S. University of Central Florida, 2011

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Department of Microbiology and Molecular Biology
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
Orlando, Florida

Summer Term
2013

Major Professor: Kenneth Teter

ABSTRACT

Cholera toxin (CT), secreted from *Vibrio cholerae*, causes a massive fluid and electrolyte efflux in the small intestine that results in life-threatening diarrhea and dehydration which impacts 3-5 million people per year. CT is secreted into the intestinal lumen but acts within the cytosol of intestinal epithelial cells. CT is an AB_5 toxin that has a catalytic A1 subunit and a cell binding B subunit. CT moves from the cell surface to the endoplasmic reticulum (ER) by retrograde transport. Much of the toxin is transported to the lysosomes for degradation, but a secondary pool of toxin is diverted to the Golgi apparatus and then to the ER. Here the A1 subunit detaches from the rest of the toxin and enters the cytosol. The disordered conformation of free CTA1 facilitates toxin export to the cytosol by activating a quality control mechanism known as ER-associated degradation. The return to a folded structure in the cytosol allows CTA1 to attain an active conformation for modification of its Gsα target through ADP-ribosylation. This modification locks the protein in an active state which stimulates adenylate cyclase and leads to elevated levels of cAMP. A chloride channel located in the apical enterocyte membrane opens in response to signaling events induced by these elevated cAMP levels. The osmotic movement of water into the intestinal lumen that results from the chloride efflux produces the characteristic profuse watery diarrhea that is seen in intoxicated individuals.

The current model of intoxication proposes only one molecule of cytosolic toxin is required to affect host cells, making therapeutic treatment nearly impossible. However, based on emerging evidence, we hypothesize a threshold quantity of toxin must be present within the cytosol of the target cell in order to elicit a cytopathic effect. Using the method of surface plasmon resonance along with toxicity assays, I have, for the first time, directly measured the efficiency of toxin delivery to the cytosol and correlated the levels of cytosolic toxin to toxin

activity. I have shown CTA1 delivery from the cell surface to the cytosol is an inefficient process with only 2.3 % of the surface bound CTA1 appearing in the cytosol after 2 hours of intoxication. I have also determined and a cytosolic quantity of more than approximately .05ng of cytosolic CTA1 must be reached in order to elicit a cytopathic effect. Furthermore, CTA1 must be continually delivered from the cell surface to the cytosol in order to overcome the constant proteasome-mediated clearance of cytosolic toxin. When toxin delivery to the cytosol was blocked, this allowed the host cell to de-activate Gsα, lower cAMP levels, and recover from intoxication. Our work thus indicates it is possible to treat cholera even after the onset of disease. These findings challenge the idea of irreversible cellular toxicity and open the possibility of post-intoxication treatment options.

I would like to dedicate this thesis to my Mom and Dad for believing in me each step of the way and supporting me whole heartedly; even though they still can only tell their friends I am trying to cure cholera.

ACKNOWLEDGMENTS

I would like to thank first and foremost Dr. Teter for giving me the chance to do research as a scared sophomore and accepting me into his lab. I would not be the driven researcher I am without his mentoring me through my growth from that day. I think of the story of Sisyphus very often and know that research is truly an uphill battle with many failures, but it's a climb that I have enjoyed and will enjoy through the extent of my research career.

I would also like to thank Helen for giving me guidance both in and outside the lab. She has had unprecedented patience in training me day in and day out. I truly owe my lab skills to her. Next I would like to thank Mike for sharing all of his SPR expertise with me having great patience with training me and analyzing data. I would like to thank Tuhina for keeping my spirits up and the food spicy. Lastly I would like to thank Neyda for supporting me through the ups and downs of the program. Without all of these invaluable people I now consider my dysfunctional family and the nontraditional lab environment we have created I would not have been able to complete this work and stay sane at the same time.

I would also like to thank the rest of my committee members: Suren Tatulian, Ph.D., Travis Jewett, Ph.D., and Antonis Zervos, Ph.D. for their continual advice and support in the completion of my thesis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS/ABBREVIATIONS

BfA	Brefeldin A
CT	Cholera toxin
DMEM	Dulbecco's Modified Eagle Medium
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
FBS	Fetal bovine serum
GA	Geldinamycin
PBS	Phosphate buffered saline
PBS-T	PBS with Tween 20
PDI	Protein disulfide isomerase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
TBS-T	Tris-buffered saline and Tween 20
TGN	Trans Golgi network

CHAPTER ONE: INTRODUCTION

Vibrio cholerae

Vibrio cholerae is a Gram negative, waterborne pathogen transmitted through a fecal-oral route. Infection occurs from ingesting food or water contaminated with the bacterium which subsequently colonizes the small intestine and releases a toxin. The resulting disease affects 3-5 million people and causes 100,000–120,000 deaths per year [1]. Although cases have been rare in industrialized nations, it is still prevalent in various areas with inadequate sanitation measures. WHO reports recent outbreaks of cholera in Haiti, Pakistan, and Central Africa and highlight the ever present presence of this disease worldwide. Infected individuals exhibit massive fluid and electrolyte efflux in the small intestine that results in life-threatening diarrhea and dehydration. One in twenty infected individuals has a severe reaction and can exhibit profuse watery diarrhea, losing up to 6 liters of fluid per day. Without proper treatment, dehydration and shock resulting in death can occur within hours [2]. Current treatment for the deadly symptoms of this disease included rehydration in the form of an oral rehydration salt solution or, in more serious cases, administration of intravenous Ringer's lactate [3]. Recovery from cholera occurs after the intoxicated enterocytes, which have a 3-5 day life span [4], are sloughed from the intestinal epithelium. Antibiotics aimed to kill the bacteria are effective but seldom available in affected areas, and emerging antimicrobial resistance to various strains is of concern [5]. As the disease results from a toxin secreted by *V. cholerae,* the toxin directly could be a potential target for therapeutic development.

Cholera Toxin

Cholera toxin (CT), secreted from *V. cholerae*, causes a massive fluid and electrolyte

efflux in the small intestine that results in life-threatening diarrhea and dehydration. *V.choerae* colonizes the lining of the small intestine and begins to produce CT once it has safely passed the acidic pH of the stomach. CT is the main virulence factor of *V. choerae* and is released from the bacterium into the extracellular environment [6].CT in the medium must bind to and enter the host cell in order reach its Gsα target within the cytosol of the cell. In order to reach the cytosol, translocation from the endoplasmic reticulum (ER) is required. This occurs after CT has been transported in a retrograde manner from the cell surface to this location [7].

CT is an AB_5 toxin which has a catalytic A1 subunit and a homopentameric cell binding B subunit. The A chain is proteolytically nicked to produce a disulfide-linked A1/A2 heterodimer. The A2 subunit and B pentamer also maintain non-covalent interactions with CTA1 which secure the A1 subunit to the rest of the toxin [8].

Retrograde Transport

Retrograde transport is a normal cellular process that moves cargo from the plasma membrane to the ER with the aid of vesicle carriers. CT uses this retrograde transport to move from the cell surface to the ER on the path to its target in the cytosol. CT, due to its lack of pore forming ability, must utilize channels in the ER to move into the cytosol. The toxin first binds to the GM1 ganglioside receptors on the surface of intestinal epithelial cells and is subsequently internalized by a clathrin-independent pathway. The toxin is directed to the early endosomes, with a majority of internalized toxin transported to the lysosomes for degradation. A lesser amount moves to the Golgi apparatus and then to the ER. The resident redox state of the ER leads to reduction of the CTA1/CTA2 disulfide bond. As determined by reduction of the CTA1/CTA2 disulfide bond which occurs in the ER, only 3-10% of surface bound toxin reaches

the ER after 1-2 hours of exposure [9-12]. Dissociation of reduced CTA1 from CTA2/CTB_5 does not occur spontaneously but requires the aid of chaperones. Once freed from the rest of the toxin, the CTA1 polypeptide can pass through an ER translocation pore to the cytosol. The efficiency of this delivery to the cytosol has never been determined.

Toxin Translocation from the ER to the Cytosol

The isolated CTA1 subunit is a disordered, thermally unstable protein. It assumes a disordered conformation after separation from the holotoxin in the ER, and regains an ordered conformation after entry into the cytosol. The disordered tertiary conformation of free CTA1 facilitates toxin export to the cytosol by activating a quality control mechanism known as ER-associated degradation (ERAD). This pathway is generally used to expel misfolded or misassembled secretory proteins from the ER for degradation in the cytosol. Unfolded CTA1 mimicks an unfolded substrate and is able to thus reach the cytosol using ERAD to move through a protein-conducting channel in the ER membrane. Terminally misfolded proteins are targeted to a translocon by a complex of chaperones [13].

Drugs with known functions such as sodium 4-phenylbutyrate (PBA), glycerol, and geldanamycin (GA) have been used to block defined steps in the CTA1 intoxication [4,14,33]. PBA and glycerol both block the unfolding of dissociated CTA1 to a translocation competent state and thus prevent CTA1 export from the ER to the cytosol, while GA prevents the chaperone mediated refolding of CTA1 once in the cytosol. Since PBA is an FDA-approved drug for the treatment of urea cycle disorders and GA is being used in clinical trials as an anti-cancer agent, they could possibly be used to treat cholera [14].

Toxin Extraction from the ER by Hsp90

Unfolded or misfolded ERAD substrates are exported out of the ER via protein-conducting channels [16-17]. Host cytosolic factors must produce the driving force for substrate extraction from these ER-specific channels. ERAD processing involves multiple host chaperones, proteins that assist in protein folding and/or unfolding and the assembly or disassembly of macromolecular structures. These chaperones prevent the aggregation of misfolded proteins in the ER and can direct ERAD substrates to the proteasome for degradation. In the case of CTA1, chaperones assist the refolding of CTA1 into an active conformation once it has entered the cytosol. Chaperones are required to stabilize and/or refold the toxin because its thermal instability at physiological temperature leaves it in a disordered state. Most ERAD substrates are extracted from the ER through a pathway that involves p97 [18]. However, p97 has been determined not to play any role in CTA1 dislocation [19,20].The cytosolic chaperone Hsp90 helps extraction from the ER [21] and it has been shown that it is required for CTA1 passage into the cytosol. Hsp90 bound to CTA1 in an ATP-dependent manner was blocked by geldanamycin (GA), an established Hsp90 inhibitor. GA has been shown to inhibit the chaperone Hsp90-mediated refolding of CTA1 in the ER as well as block intoxication in cultured cells and fluid accumulation when used in rabbit ileal loop studies. GA is currently in clinical trials as an anti-cancer agent. GA thus represents a promising therapeutic for the treatment of cholera.

Toxin Degradation in the Cytosol

The cell itself has a multitude of mechanisms, such as the previously mentioned lysosome, to reduce the level of waste proteins. Within the cytosol, the proteasome is in place to reduce the level of misfolded proteins. After export to the cytosol, ERAD substrates are

ubiquitinated to target them for degradation by the 26S proteasome [15-16, 22-23]. The proteasome is a cylindrical complex containing central barrel shaped pore known as the 20S "core" and two 19S "cap" that recognizes the polyubiquitin tags and initiates the degradation process with ATP-depending unfolding [24]. CTA1 avoids degradation by the 26S proteasome because it contains only two lysine residues that are normally the sites of ubiquitin conjugation [25]. However, when CTA1 is unfolded it is susceptible to ubiquitin-independent degradation by the 20S proteasome. This variant of the proteasome consists of only the catalytic core of the 26S proteasome, thus lacking the ubiquitin recognition and ATP-dependent activities of the 19S cap seen in the 26S proteasome [26]. The Teter lab has further shown that the cytosolic pool of CTA1 is degraded by this mechanism with a half-life of 2 hours, and the use of proteasome inhibitors increased this time. These results suggest the amount of cytosolic toxin is proportional to the amount of toxicity.

Toxin Activity in the Cytosol

Once in the cytosol, CTA1 attains an active refolded conformation with the aid of chaperones such as Hsp90. Refolded CTA1 activates the stimulatory α subunit of the heterotrimeric G protein which is located in lipid rafts at the cytoplasmic face of the eukaryotic plasma membrane [27]. CTA1 modifies its Gsα target by ADP-ribosylation. Gsα is a molecular switch that is regulated by GTP binding and hydrolysis. Catalytically active CTA1 fixes Arg^{201} of Gsα with ADP-ribose which prevents its ability to hydrolyze bound GTP and thus locks it in an active state. This results in overstimulation of adenylate cyclase, causing an increase in the production of the secondary messenger cAMP above normal levels. If CT intoxication is not halted, the subsequent dysregulation of cAMP levels leads to the opening of the cystic fibrosis

transmembrane conductance regulator (CFTR) channel in the apical plasma membrane of the intestinal epithelial cell. This, in turn, stimulates chloride secretion and ultimately leads to an efflux of water into the lumen of the small intestine [28]. The osmotic movement of water produces a diuretic response which is responsible for the rapid and extreme dehydration that is seen in intoxicated individuals.

Reversal of Toxin Activity

In addition to the ability to degrade CTA1 directly, the cell has mechanisms in place to reverse the toxin-activated signaling pathway. The host cell has a multitude of mechanisms in place to regulate and degrade common cytosolic events and components. The host ADP-ribosyl(arginine)protein hydrolase will catalyze the removal of the ADP-ribose moiety from the locked, active G protein [29]. This will allow Gsα to turn off by hydrolyzing its bound GTP. There is also a rapid turnover of ADP-ribosylated Gsα by the proteasome [30]. This substantially reduces the amount of ADP-ribosylated Gsα in the cell. Phosphodiesterases are able to degrade cAMP and can reduce concentrations back to basal level. With these mechanisms in place, we hypothesize CTA1 must be continually delivered to the cytosol in order to overcome the constant cellular pressures which degrade CTA1 and reverse its activity. This is contrary to the current paradigm, described below, that only one molecule of cytosolic toxin is required to irreversibly affect host cells [31-32]. The cell's ability to degrade CTA1 and reverse toxin activity is indicative of the requirement of more than one molecule for intoxication and consistent with results that show levels of cytosolic toxin proportional to the level of toxicity. Current treatments do not sufficiently block translocation of CTA1 to the cytosol and the fight between entry and degradation favors toxicity over reversal by the cell. New therapeutics that may more

efficiently block translocation may be able to lower the quantity of toxin to a level at which the balance may favor the cell's innate mechanisms. Thus, it is theoretically possible to reverse effects of cholera intoxication.

Current Model

It is widely thought that AB toxins such as CT are essentially so potent that it only takes one to a few molecules of cytosolic toxin to generate a toxic effect. Furthermore, the cellular effects of CT are still considered irreversible. Thus, the dominant model views the diarrhea from cholera as an irreversible event. It is thought that individuals only recover from cholera when the intoxicated cells are sloughed from the epithelial monolayer. This model of intoxication implies the idea of a post-intoxication therapeutic treatment is nearly impossible.

Hypothesis

We hypothesize a threshold quantity of toxin must be present within the cytosol of the target cell in order to elicit a cytopathic effect. Our model contradicts the currently accepted theory that one molecule of toxin is enough to induce toxicity in the cell. Previous studies provided only indirect measures of cytosolic toxin with a high level of error and did not address the relationship between toxin quantity and toxin activity within the cytosol [39-40]. With recent advances in technology, we can now detect cytosolic CTA1 using surface plasmon resonance (SPR) [4,14,33-35]. This SPR assay is an innovative way to look at precise CTA1 cellular content at levels too low to be previously detected. This assay will allow us to draw correlations between the levels of cytosolic CTA1 to the extent of intoxication and could change the paradigm of how we view the intoxication process. Using this method we have been able to measure the efficiency of toxin delivery to the cytosol and correlated the levels of cytosolic toxin

to toxin activity. I have shown only 2.3 % of the surface bound CTA1 appeared in the cytosol after 2 hours of intoxication. I have also determined and a cytosolic quantity of more than .05ng of cytosolic CTA1 must be reached and maintained in order to elicit a cytopathic effect and overcome the constant proteasome-mediated clearance of cytosolic toxin. Blocking toxin trafficking allowed the host cell to de-activate Gsα, lower cAMP levels, and recover from intoxication. Our work thus indicates it is possible to treat cholera even after the onset of disease and challenges the idea of irreversible cellular toxicity while opening the possibility of post-intoxication treatment options.

CHAPTER TWO: METHODOLOGY

Chemicals and Reagents

Sigma Aldrich (St. Louis, MO)

- Ethylenediaminetetraacetic Acid (EDTA)
- Protease Inhibitor Cocktail
- Tetramethylethylenediamine (TEMED)
- Monosialoganglioside GM1 from bovine brain,
- 3-Isobutyl-1-Methylxanthine (IBMX)
- Digitonin
- Brefeldin A
- ALLN
- Glycerol

Fisher Scientific (Pittsburgh, PA)

- 2-Mercaptoethanol (β-ME)
- Ethanol 200 proof (EtOH)
- Gel Code Blue Stain Reagent
- Glycerol
- Methanol (MeOH)
- Sodium Chloride (NaCl)
- Sodium Hydroxide (NaOH)
- Sodium Phosphate Dibasic Anhydrous (Na2HPO4)

- Sodium Phosphate Dibasic Heptahydrate ($Na_2HPO_4 \cdot 7H_2O$)
- Sodium Phosphate Monobasic Anhydrous (NaH_2PO_4) Sodium
- Phospate Monobasic Monohydrate ($NaH_2PO_4 \cdot H_2O$) Tris Base
- Tryptone
- Tween-20

Stressgen (Farmingdale, NY)

- Geldanamycin

Invitrogen (Carlsbad, CA)

- Antibiotic-Antimycotic
- Dulbecco's Modified Eagle Medium (DMEM)
- Ham's F-12
- Trypsin/EDTA

Amresco (Solon, OH)

- Ammonium Persulfate (APS)
- Bromophenol Blue
- Glycine
- HEPES
- Methionine
- Sodium Dodecyl Sulfate (SDS)
- Tris-Cl
- Triton X-100

Calbiochem (La Jolla, CA)

- Digitonin
- Sodium 4-phenylbutyrate (PBA)

Bio Rad (Hercules, CA)

- 40% Acrylamide /Bis Solution
- Extra Thick Filter Paper

Atlanta Biologicals (Lawrenceville, GA)

- Fetal Bovine Serum

Pierce Biotechnology, Inc (Rockford, IL)

- N-hydroxysuccinimide (NHS)

Thermo Scientific (Waltham, MA)

- 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC)

Gibco (Grand Island, NY)

- DMEM
- Antibiotic-Antimycotic

GE Healthcare (Piscataway, NJ)

- ELISA cAMP kit
- Western blotting detection reagents

Toxins

List Biological Laboratories (Campbell, CA)

- Cholera Toxin, Holotoxin

Calbiochem (La Jolla, CA)

- Cholera Toxin, A subunit

Antibodies

Stressgen (Ann Arbor, MI)

- Rabbit α-Hsp90
- Rabbit α-PDI

Sigma Aldrich (St. Louis, MO)

- Rabbit α-CTA

Jackson Immunoresearch (West Grove, PA)

- Goat anti-Rabbit IgG conjugated to HRP

Equipment

Bio Rad (Hercules, CA)

- Bio Rad Power Pac Basic
- Bio Rad Power Pac HC
- Trans-Blot SD Semi-Dry Transfer Cell

BioTek Instruments (Winooski, VT)

- Synergy 2 Plate Reader

Other Materials

Grenier Bio One

- 6-well flat-bottom tissue culture plates
- 24-well flat-bottom tissue culture plates

American Type Culture Collection

- Chinese Hamster Ovary (CHO) cells
- HeLa cells

Buffers

Phosphate Buffered Saline (PBS), 10x

- 82.3 g Na_2HPO_4 (0.58M)
- 23.5 g NaH_2PO_4 (0.17M)
- 40 g NaCl (0.69M)
- H_2O to 1 liter

Tris-Buffered Saline (TBS)

- 10x 24.24 g Tris-Cl
- 5.56 g Tris Base
- 80.1 g NaCl
- H_2O to 1 liter

Transfer Buffer

- 100 ml 10x SDS-Electrophoresis Running Buffer
- 200 ml MeOH
- 600 ml H2O

SDS-Electrophoresis Running Buffer, 10x

- 30.2 g Tris Base
- 144 g Glycine
- 10 g SDS H2O to 1 liter

SDS Sample Buffer, 4x

- 50 ml 4x Tris-Cl/SDS pH 6.8
- 40 ml Glycerol
- 8 g SDS
- 2 mg bromophenol blue
- 100 mM 2-mercaptoethanol (7µl of 14.3 M ß-ME /ml of 4x buffer)

HCN Buffer

- 50 mM HEPES, pH 7.5
- 150 mM NaCl
- 2 mM CaCl2
- 10 mM N-ethyl maleimide (NEM)
- 1:20 dilution of Protease Inhibitor Cocktail

Cell Culture

HeLa cells were grown in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic at 37°C and 5% CO_2 unless otherwise indicated.

Detection of Cytosolic CTA1

HeLa cells were seeded in triplicate to 6-well plates in complete DMEM medium to achieve an 80% confluent monolayer after an overnight incubation. Cells were incubated in serum free DMEM containing 100 ng/mL of ganglioside GM1 for 1 h at 37°C. GM1 receptor is added to the medium because HeLa cells do not normally express this receptor but will incorporate it from the medium into the plasma membrane. After washing with phosphate buffered saline (PBS) to remove excess GM1, the cells were subsequently incubated for 30 min at 4°C with DMEM containing 1 µg/ml of CT. After this incubation, the cells were washed twice with PBS to remove unbound CT. At this point, surface bound CT can be determined via digitonin permeabilization and SPR analysis (as described below) of the pellet fraction containing surface bound CT. The washed cells were placed in toxin-free/serum-free media and returned to 37°C for various time points and treatments as indicated. Only when the plates are moved to the 37°C incubator is the toxin internalized; at this stage the chase interval begins. After washing with PBS at the end of each chase point, digitonin permeabilization was performed to separate cytosolic and outermembrane fractions to be used to analyze the cytosolic content of CTA1 by SPR. This process was begun at the end of each chase interval as well as at the end of the 4°C pulse labeling by lifting cells from the 6-well plate using a 5 minute 4°C incubation with 400 µl of 0.5 mM ethylenediaminetetraacetic acid (EDTA) in PBS. Triplicate wells were collected into a single microcentrifuge tube and spun at 5,000× g for 5 min. The

supernatant was discarded and the cell pellet was incubated on ice for 10 minutes. The cell pellet was subsequently resuspended in 100 µl of 0.04% digitonin in HCN buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM CaCl2, 10 mM N-ethylmaleimide, and a protease inhibitor cocktail) for 10 minutes at again on ice. The digitonin-permeabilized cells were then spun at 16,000 × g for 10 min at room temperature, after which the supernatant (i.e., cytosolic fraction) was collected and placed in a fresh microcentrifuge tube for SPR analysis. The membrane pellet was collected from the pulse sample to determine total CTA1. Well specific cell counts were recorded for each experiment with the use of a hemocytometer.

SPR Analysis

Experiments were performed with a Reichert (Depew, NY) SR7000 surface plasmon resonance (SPR) refractometer. The flow rate for all steps was 41 µl/min. For experiments involving antibody-coated plates, an EDC-NHS activation buffer with 0.08 mg/ml of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and 0.02 mg/ml of N-hydroxysuccinimide (NHS) mixed in a 1:1 ratio was perfused over a Reichert gold-plated glass slide for 5 min. A 5 min wash with 10 mM PBS containing 0.05% Tween 20 (PBST) pH 7.4 was used to remove the activation buffer, after which an monoclonal anti-CTA1 antibody at 1:2000 dilution in 20 mM sodium acetate, (pH 5.2) was perfused over the slide for 5 min. Unbound antibody was removed with a 5 min PBST wash, and the remaining active groups on the sensor slide were deactivated with a 5 min exposure to 1 M ethanolamine (pH 8.5). PBST was perfused over the slide for 5 to 10 min to establish a stable baseline signal corresponding to the mass of the sensor-bound antibody. Supernatant samples from the digitonin-permeabilized cells were brought to a final volume of 1 ml in PBST pH 7.4, and pellet samples were brought to final

volume of 1ml in PBST containing 1% Triton X-100. These samples were then perfused over the sensor slide coated with an anti-CTA1 antibody. To establish a standard curve CTA standards were diluted in PBST pH 7.4 to appropriate concentrations (500, 100, 10, 1, 0.1, 0.01, 0.001 ng/ml) and flowed over the CTA1 sensor slide for 5 min each, followed by a 5-min PBST wash. The association rate constants for the CTA standards were plotted as a function of protein concentration, and the slope of the resulting standard curve was then used to calculate the quantity of CTA1 in experimental samples. Reichert LabView software was used for data collection. The BioLogic (Campbell, Australia) Scrubber 2 software and WaveMetrics (Lake Oswego, OR) Igor Pro software were used to analyze the data and generate figures. Binding affinities between CTA1 and the antibody bound plate were calculated in the Scrubber 2 software.

Western Blot

For Western blot analysis, pellet and supernatant fractions were produced for digitonin-permeabilized as described above. 120 µl of 1× sample buffer was added to the pellet and 20 µl of 4× sample buffer was added to the supernatant. Equivalent 25 ul volumes of each sample were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 15% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane. The rabbit anti-Hsp90 antibody was used at a 1:5,000 dilution and the rabbit anti-PDI antibody was used at a 1:5,000 dilution for an overnight incubation at 4°C. The secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was used at a 1:20,000 dilution for a 30 minute room temperature incubation. ECL Plus Western blotting detection reagents were used for protein

detection according to the manufacturer's instructions. A blot was run to detect the presence of the previously mentioned target proteins.

Toxicity Assay

HeLa cells as described in the above CT detection assay were also seeded and treated in parallel to SPR and were used to detect cytotoxicity as values of cAMP. 6-well plates were incubated overnight until 80% confluent. Cells were pretreated with GM1 and exposed to CT as described above. At the end of the chase interval, the cells were washed with PBS and incubated with 0.5 ml of ice-cold HCl-ethanol (1:100) for 15 min at 4°C. Supernatants were placed in microcentrifuge tubes and allowed to air dry. cAMP levels were then determined using an ELISA cAMP competition assay kit as per manufacturer instructions. The basal level of cAMP from equivalent numbers of unintoxicated cells was also determined. All conditions were performed in triplicate.

Calculation of Cytosolic CTA1 Levels

To calculate the precise amount of cytosolic CTA1 observed in the cell, CTA standards were perfused over each SPR plate used to detect cytosolic CTA1. Using the Ka values from these standards in comparison to the Ka values from out experimental samples, we could back-calculate the amount of CTA1 within the cytosol. With the use of a hemocytometer, cells counts from each corresponding experiment were carried out and used to determine the amount of CTA1 per cell.

CHAPTER THREE: RESULTS

To determine the efficiency of CTA1 transport to the cytosol, we used digitonin permeabilization to collect and SPR to quantify the amount of CTA1 in the cytosol.

HeLa cells treated with GM1 were exposed to toxin 1 μg/ml of CT at 4°C.Toxin can bind to the cell surface at 4°C but is not endocytosed. At this point, the total cell-associated amount of CT can be determined via digitonin permeabilization and SPR analysis of the pellet fraction containing surface bound CT. For quantification of cytosolic CTA1, cells placed at 37°C for various time points with or without drug treatments. Only when the plates are moved to the 37°C incubator is the toxin internalized; at this stage the chase interval begins. After each chase point, digitonin permeabilization was performed to separate cytosolic and membrane fractions to be used to analyze the cytosolic content of CTA1 by SPR. 0.5 mM ethylenediaminetetraacetic acid (EDTA) in PBS was used to collect cells from the plate. The cell pellet was subsequently resuspended in 100 μl of 0.04% digitonin in HCN buffer for 10 minutes on ice. The digitonin-permeabilized cells were then spun at after which the supernatant (i.e., cytosolic fraction) was collected and placed in a fresh microcentrifuge tube for SPR analysis. PBS was used to bring the final volume of each cytosolic fraction to 1ml. Cell counts were recorded for each experiment with the use of a hemocytometer.

To verify proper digitonin permeabilization generated clean cytosolic and organelle fractions, both supernatant and pellet fractions collected from HeLa cells were run on SDS-PAGE gels. Western blots against known cytosolic (Hsp90) and ER lumen (PDI) proteins were used to confirm the reliability of the fractionation process by determining the distribution of these proteins. Due to their known localization, Hsp90 should only be detected within the supernatant fraction and PDI within the pellet fraction if fractionation was successful.

Supernatant and pellet fractions from two independent experiments were used to demonstrate reproducibility. Anti-Hsp90 and anti-PDI blots each detected only a single band at the corresponding molecular mass and in the respective supernatant and pellet fraction indicating accurate fractionation.

SPR can be used to detect protein-protein interactions as well as test for the presence of a particular protein within a sample. For these experiments, a protein of interest is perfused in microliter quantities over a gold and glass sensor slide that is coated with either a second protein of interest or an antibody to the first protein. An interaction between the two proteins increases the mass on the sensor slide, and this generates a change in the angle of reflected light which is recorded as a refractive index unit (RIU) [35].

Cytosolic fractions from intoxicated cells were run over an anti-CTA1 SPR slide as described previously. The cytosolic fractions from unintoxicated and 5ug/ml BFA-treated cells were used as negative controls to ensure the fractionation procedure did not rupture the collected organelles. BFA causes deregulation of membrane traffic through disassembly of the Golgi complex and thus prevents toxin delivery to the ER translocation site. If contamination was present, CTA1 would be detected in the cytosolic fraction of the unintoxicated or BFA treated cells. However, Figure 2 shows no detection of CTA1 in these control cells. There is also no detectable CTA1 within the cytosol of intoxicated cells after 15 minutes of toxin treatment indicating that delivery from the cell surface to the cytosol takes a greater period of time to occur. CTA1 was clearly seen within the cytosol after a 30 minute intoxication, with increasing amounts of toxin appearing in the cytosol at later 60 and 120 minute chase intervals. CTA1 standards were also perfused over the anti-CTA1 SPR slide. The Ka values determined for these standards were then plotted as a function of protein concentration (Fig 3). The slope of the

resulting standard curve was then used to calculate the quantity of CTA1 in experimental samples in Table 1. It was determined that after 30, 60 and 120 minutes .07ng, .12ng and .65ng of CTA1 is present within the cytosol respectively. Furthermore, hemacytometer counts for each experiment documented the number of collected cells and allowed us to determine the average number of CTA1 molecules inside the cytoplasm of an individual cell. This was determined to be 9450, 16136, and 87832 molecules after 30, 60, and 120 minutes of chase respectively. This was then compared to the amount of surface bound-CT at the beginning of the experiment and is presented as a percentage of the initial cell-associated toxin and determined to be .25%, .43% and 2.3% respectively. This work represents both the first direct calculation of toxin levels in the cytosol of a host cell as well as the first calculation of efficiency of transport to the cytosol where the toxin is active.

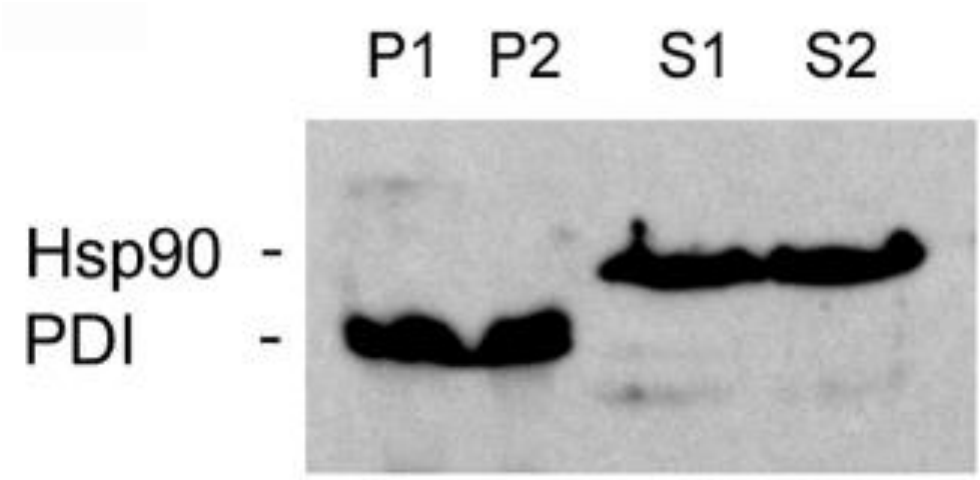

Figure 1. Proper collection of membrane and cytosolic bound fractions

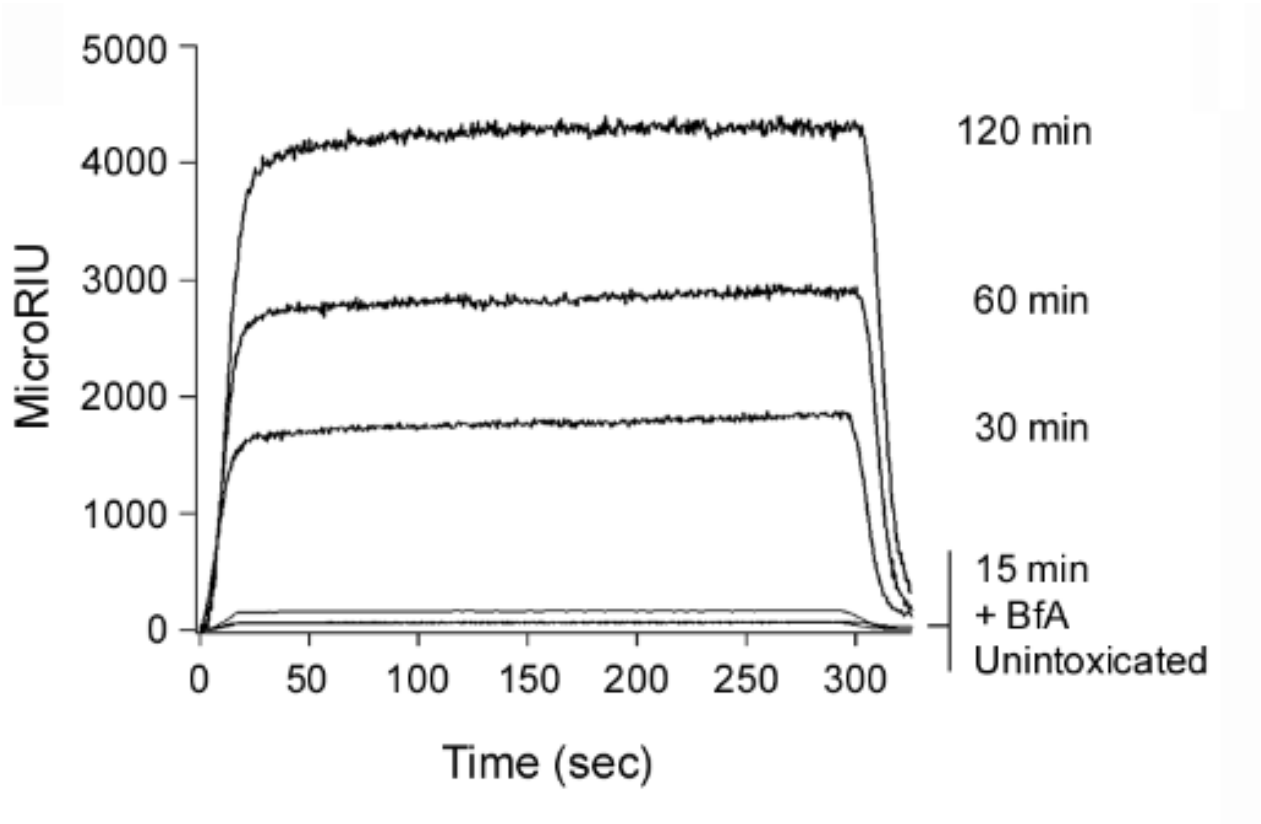

Figure 2. CTA1 is detected within the cytosol at after 30 minutes.

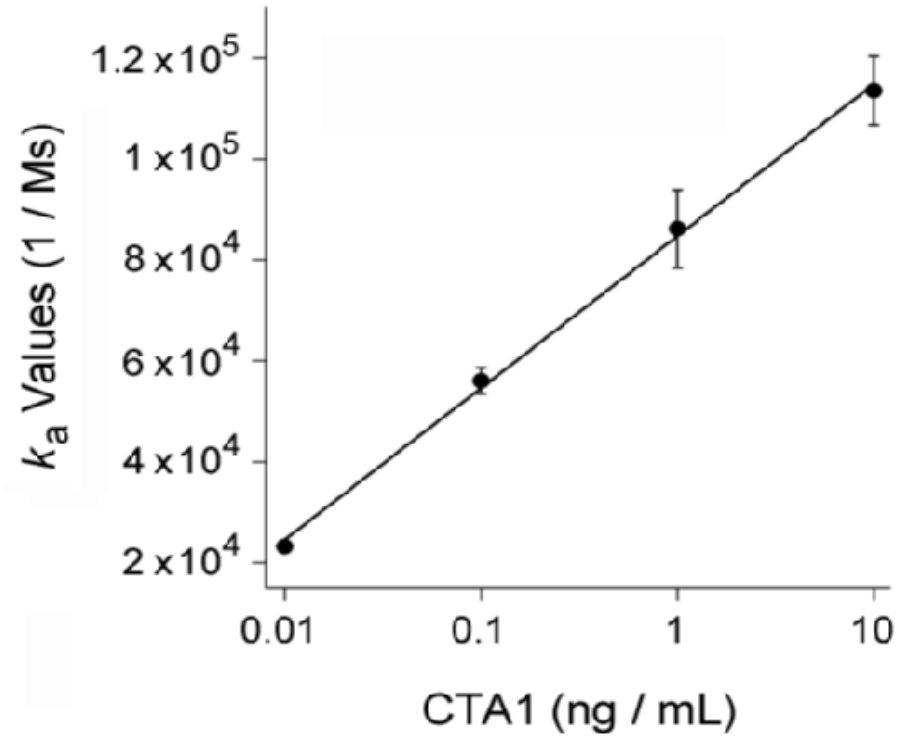

Figure 3. Ka's plotted as a function of protein concentration can be used to determine cytosolic CTA1 quantities.

Table 1. Efficiency of toxin delivery to the cytosol

Chase condition	ng cytosolic CTA1	CTA1 molecules per cell	% surface-associated CTA1
30 min	0.07 ± 0.03	9,454	0.25%
60 min	0.12 ± 0.06	16,137	0.43%
120 min	0.65 ± 0.16	87,832	2.3%

The next step was to correlate the level of toxin in the cytosol with toxin activity by examining intracellular cAMP levels in a time-dependent manner. Pulse-chase experiments were carried out as described in the aforementioned translocation assay. Pulse sample with no chase were used to determine background resting levels of cAMP activity. Intoxicated cells were treated with acidic ethanol to lyse the cells. cAMP levels were then determined using an ELISA cAMP competition assay kit as per manufacturer instructions (Figure 4). Cells were exposed to CT in the absence (circles) or presence (squares) of 3-isobutyl-1-methylxanthine (IBMX), a cAMP phosphodiesterase inhibitor that prevents the degradation of cAMP. IBMX- treated cell had greater levels of cAMP then untreated cells at 60 and 120 minutes of chase, although there was still no response above background at 30 minutes for either untreated or IBMX- treated cells. This indicated those host cAMP phosphodiesterases can partially limit the cytopathic effect of CT. Results shown in Figure 4 demonstrate a cAMP response is not present at the 30 minute chase even though toxin is seen in the cytosol at 30 minutes (Fig 2, Table 1). This result suggests the amount of toxin in the cytosol at 30 minutes of chase was not sufficient for a cAMP response. We see here the suggestion of a threshold quantity of cytosolic CTA1 may be required to elicit a toxic effect.

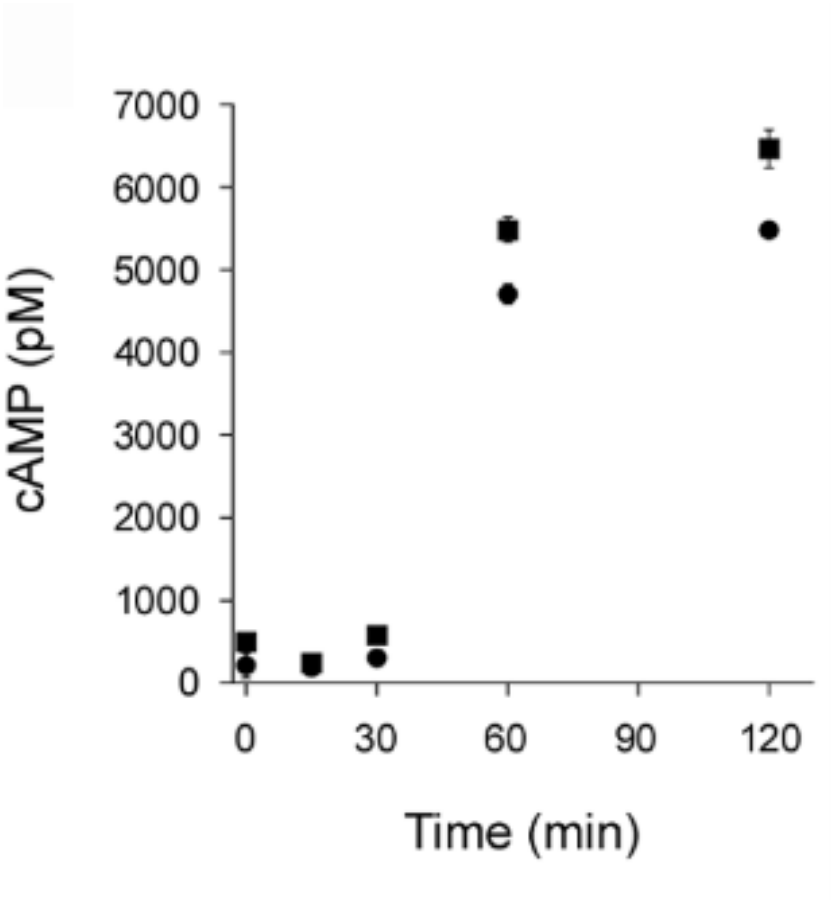

Figure 4. Toxin activity lags behind toxin appearance in the cytosol

This delay between CTA1 appearance in the cytosol and CTA1 activity in the cytosol could reflect a kinetic requirement for cytosolic CTA1 to reach and activate its Gsα target. To examine this possibility, we repeated our pulse-chase translocation assay with drug-treated cells. .1uM GA and .1mM PBA were used to restrict CTA1 access to the cytosol by disrupting the ER-to-cytosol export of CTA1. GA inhibits the Hsp90-driven extraction of CTA1 from the ER, while PBA prevents the unfolding of CTA1 to a translocation-competent state, while neither drug inhibits toxin transport to the ER. Figure 5 and 6 show PBA and GA treatment are sufficient to inhibit toxin entry to the cytosol after 60 and 120 minutes respectively. Both GA and PBA are in parallel able to reduce cAMP levels in intoxicated cells even after 60 and greatly after 120 minutes. PBA and GA were then used in combination and indicate co-treatment also inhibits toxin entry to the cytosol in Figure 8. The addition of a 100uM ALLN, a known proteasome inhibitor, was then used to determine the proteasomes role in intoxication. ALLN increases CTA1 presence in the cytosol and thus plays a role in clearing the toxin from the cytosol. A combination of GA and PBA reduces cAMP levels while the addition of a proteasome inhibitor increases this level in intoxicated cells in Figure 9. The differing quantities and resulting cAMP activity are then used to hone in on the threshold quantity of toxin required to elicit cytopathic activity in the cell. Using these quantities and activity levels it is approximated that quantities above .05 ± .02 ng would be sufficient to induce cytopathic activity.

Table 2. A threshold quantity of cytosolic CTA1 is required to generate a cAMP response.

Chase condition	ng cytosolic CTA1	CTA1 molecules per cell	cAMP response
60 min			
No treatment	0.25 ± 0.18	26,390	+
+ GA	0.03 ± 0.01	3,673	-
+ PBA	0.07 ± 0.02	7,652	-
120 min			
No treatment	0.63 ± 0.32	66,577	+
+ ALLN	0.38 ± 0.06	40,198	+
+ GA	0.02 ± 0.01	2,032	-
+ PBA	0.04 ± 0.01	4,423	-
+ GA/PBA	0.02 ± 0.01	2,043	-
+ GA/PBA & ALLN	0.05 ± 0.02	5,655	+

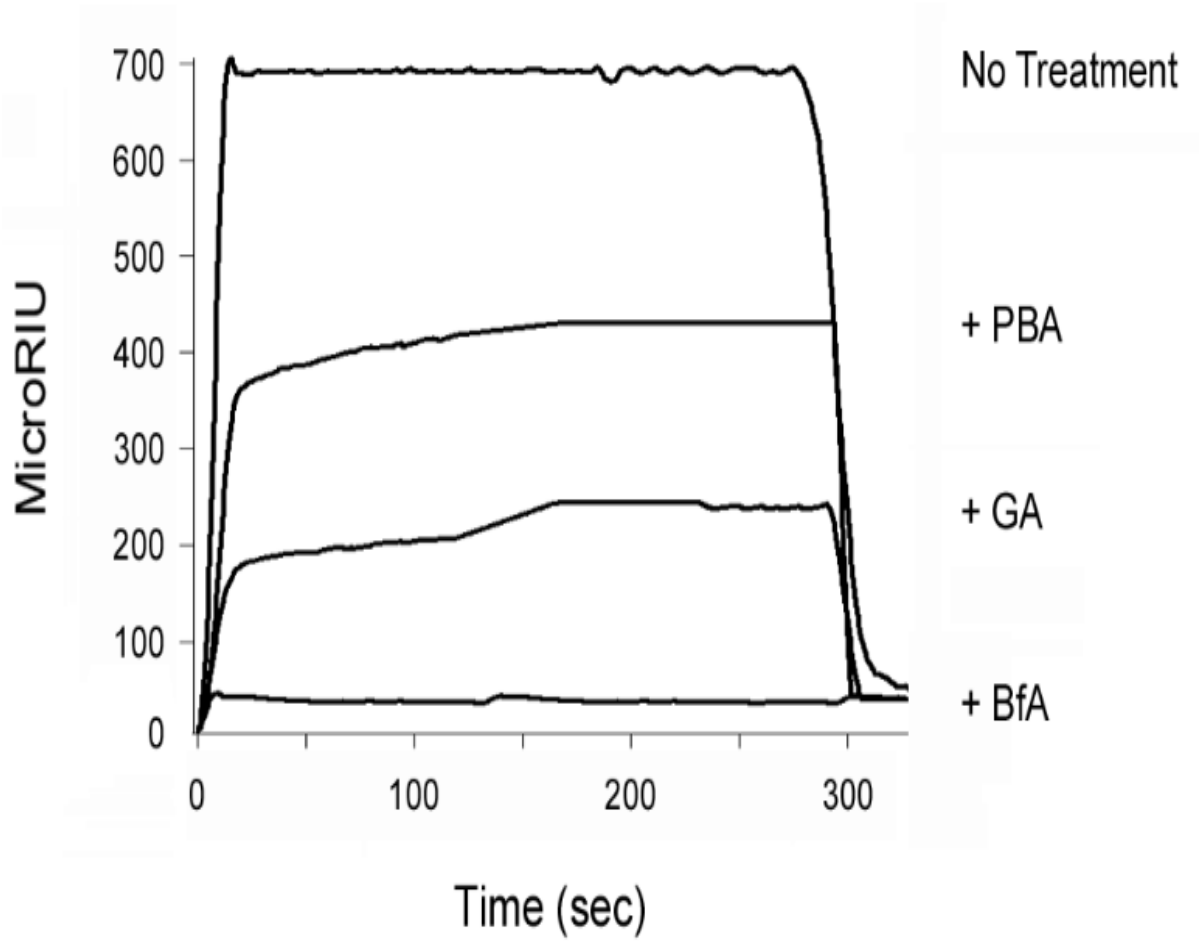

Figure 5. PBA and GA treatment are sufficient to inhibit toxin entry to the cytosol after 60 minutes.

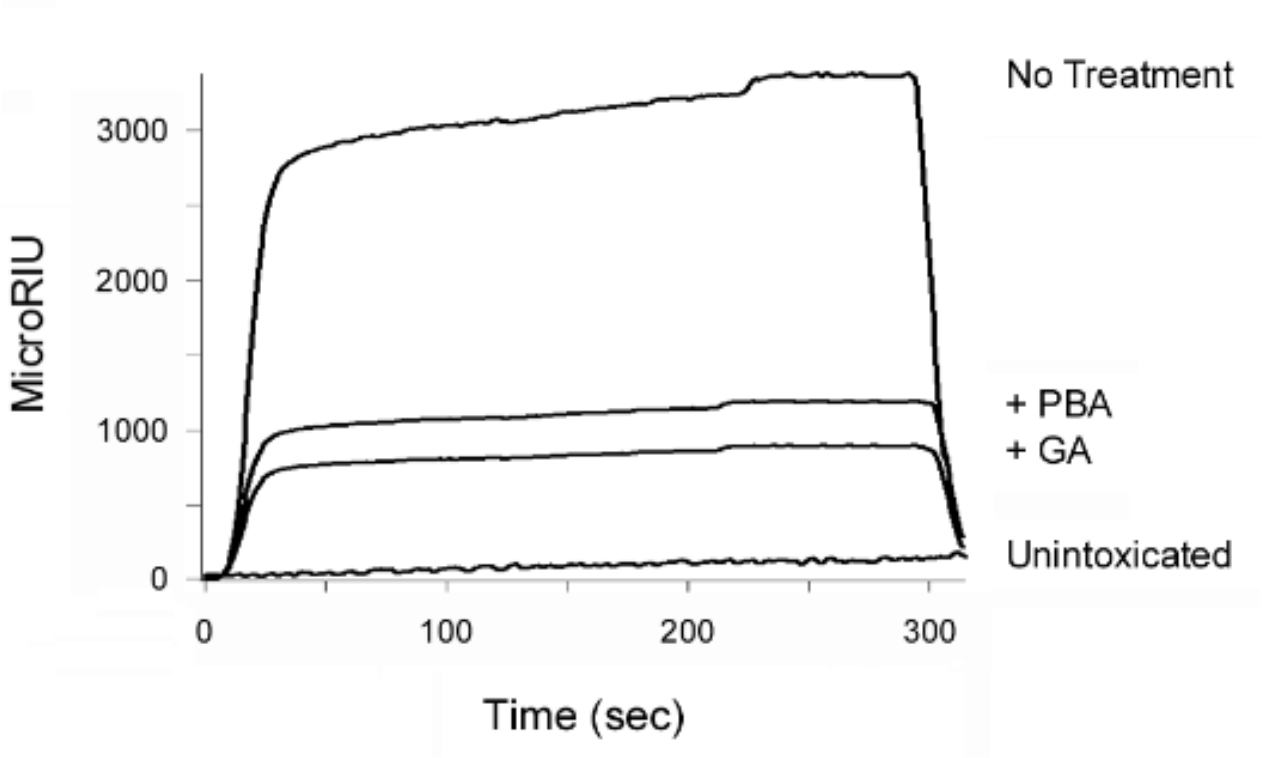

Figure 6. PBA and GA treatment greatly inhibit toxin entry to the cytosol after 120 minutes.

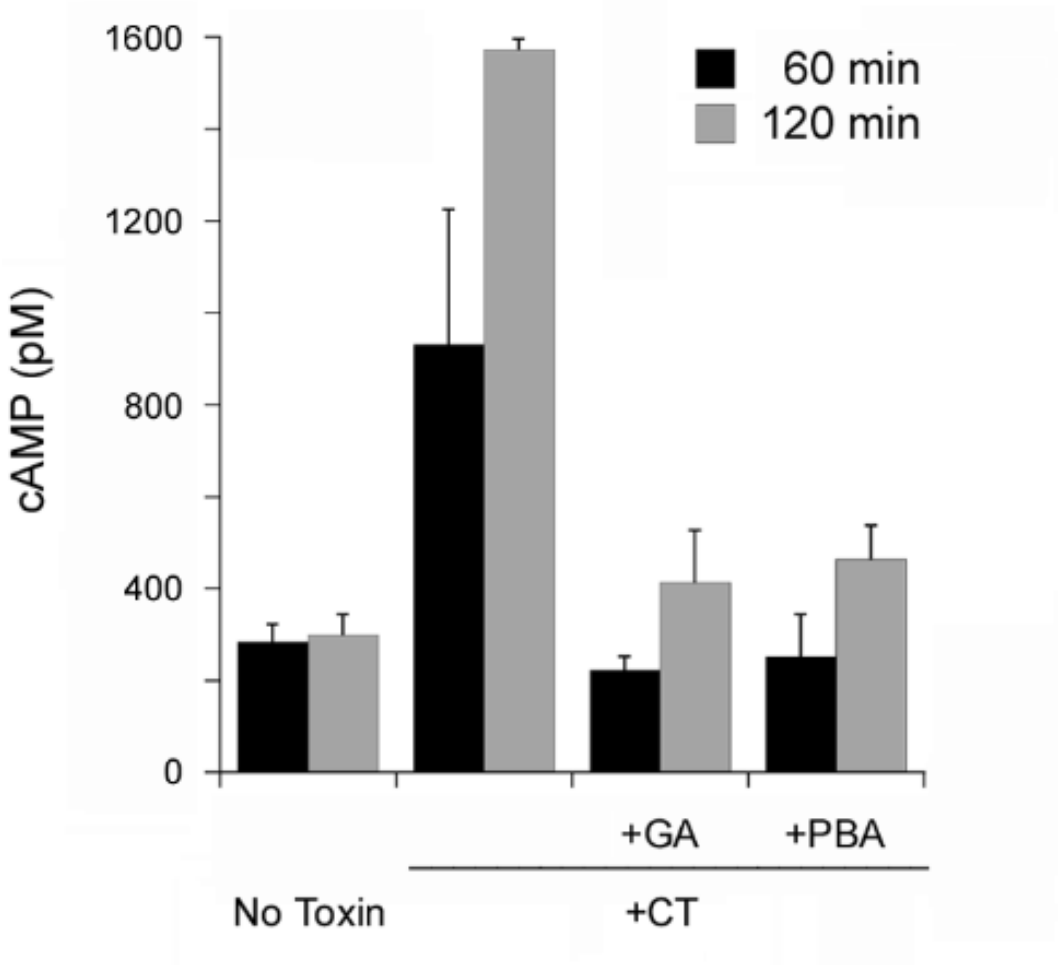

Figure 7. Both GA and PBA reduce cAMP levels in intoxicated cells after 60 and 120 minutes.

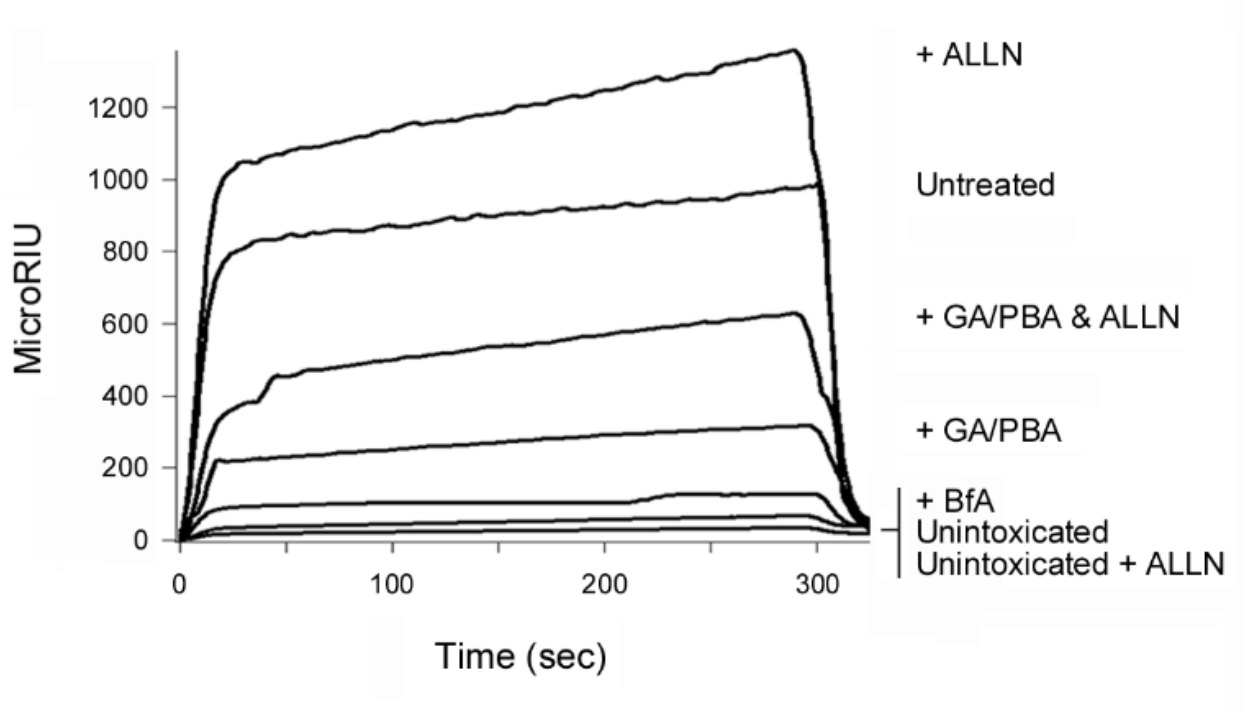

Figure 8. PBA/GA treatment inhibits toxin entry to the cytosol while the addition of a proteasome inhibitor increases its presence.

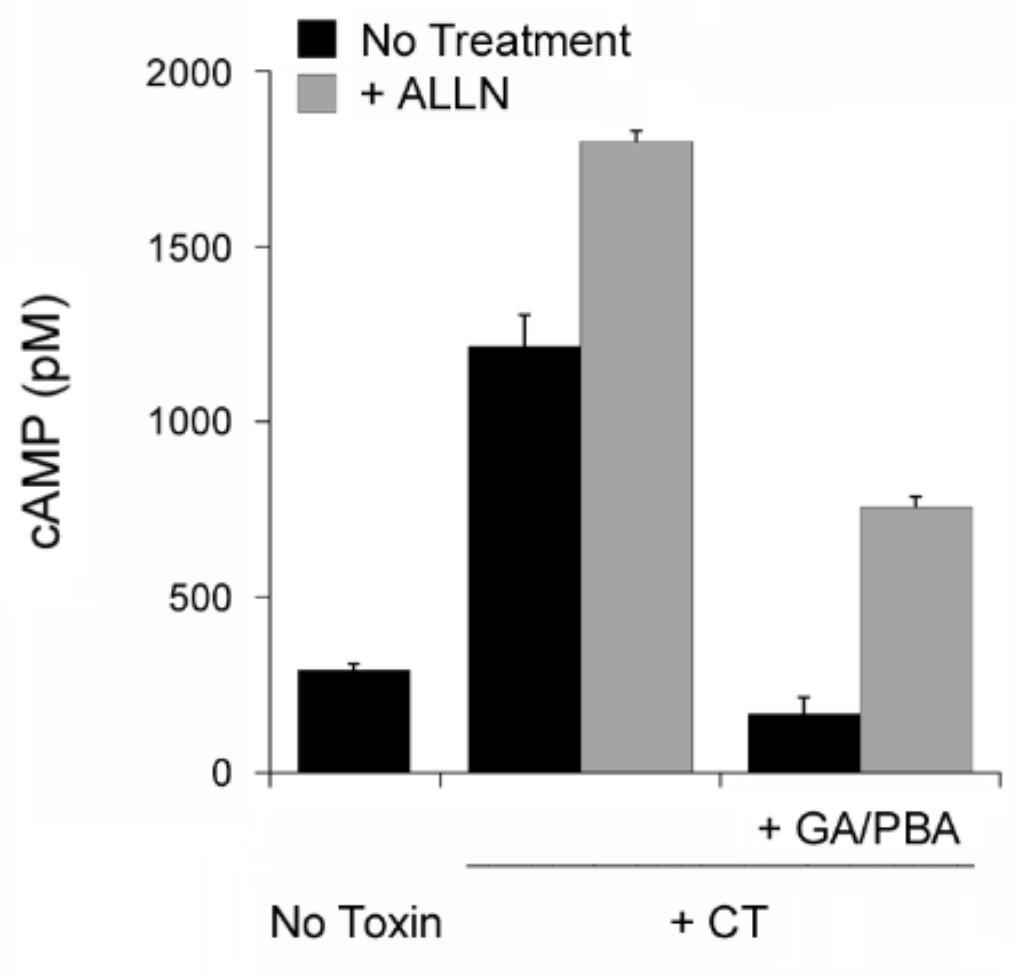

Figure 9. A combination of GA and PBA reduces cAMP levels while the addition of a proteasome inhibitor increases this level in intoxicated cells.

Figure 4 demonstrated that cAMP phosphodiesterases could limit the cAMP response to CT. We examined whether host proteasome function could also limit the cAMP response to CT. After 30 minutes of chase in our pulse-chase translocation assay, we prevented additional CTA1 from reaching the cytosol using 10% glycerol (Figure 10). This chemical chaperone prevents the unfolding and ER-to-cytosol translocation of CTA1. Using this approach, we were able to control toxin translocation. We labeled the cell surface with CT and allowed trafficking from the cell surface through the cell for 30 minutes before adding glycerol. Again, ALLN, a proteasome inhibitor, was added to a subset of glycerol-treated cells to examine the proteasome's potential role in toxin clearance from the cytosol. The amount of cytosolic toxin in untreated cells was determined after 30 and 120 minutes. The amount of cytosolic toxin in glycerol-or glycerol/ALLN- treated cells was determined after 120 minutes (drugs were applied 30 minutes into chase). Unintoxicated cells and BFA-treated cells were used as negative controls. The increase in cytosolic toxin from 30 to 120 minutes is comparable to what we have seen in that there is an increasing accumulation of CTA1 in the cytosol over time. Treatment with glycerol showed lower amounts of cytosolic toxin from 30 to 120 minutes in comparison to the untreated sample at 120 minutes. This difference was due to the block of CTA1 entry to the cytosol. If this glycerol induced block was the only factor contributing to toxicity the cytosolic quantity of toxin would be steady and equivalent to that seen at 30 minutes, but we observe a loss of signal with glycerol treatment. The loss of signal in these samples is due to toxin clearance by the proteasome. This observation is validated by the use of ALLN along with glycerol treatment. Glycerol- and ALLN-treated samples contained more cytosolic CTA1 than cells exposed to glycerol alone which indicated clearance of cytosolic toxin from glycerol-treated cells was due to proteasomal degradation. This again demonstrated proteasomal degradation has the ability to

limit the accumulation of cytosolic toxin. Proteasomal activity can lower the quantity of CTA1 when translocation to the cytosol is blocked after exposure. Overall, this suggests that CTA1 must be continuously delivered to the cytosol to overcome proteasomal degradation.

Next, we again used toxicity assays to directly correlate the level of cytosolic toxin to the extent of intoxication at 120 minutes. Figure 7 shows cells treated with glycerol treatment at 30 minutes after toxin exposure produced a cAMP response at 120 minutes chase that was barely above the basal cAMP level from unintoxicated cells. This non-response corresponded to .01 ng CTA1 present in the cytosol (Table 3). A greater quantity of .06 ng of cytosolic toxin was obtained from cells exposed to both glycerol and ALLN, which resulted in a strong cAMP response and is constant with the results seen with the cellular quantity derived from the previous data (Figure 11). However, less CTA1 was in the cytosol of glycerol/ALLN-treated cells at 120 minutes of total chase than was present in the cytosol of untreated cells at 30 minutes post-intoxication (Table 3). The lack of cAMP response after 30 minutes of chase thus indicated there is a kinetic barrier, as well as a threshold requirement, for productive intoxication.

The need to reach a threshold quantity of cytosolic toxin for a cAMP response implied that intoxication could be reversed if the level of cytosolic toxin dropped below the threshold quantity. Our data further indicated the cytosolic toxin can be removed by proteasomal activity.

Table 3. Glycerol prevents further CTA1 translocation and proteasomal activity removes CTA1 from the cytosol.

Chase condition	ng cytosolic CTA1	CTA1 molecules per cell	cAMP response
30 min			
No treatment	0.02 ± 0.005	2,103	-
120 min			
No treatment	0.66 ± 0.19	93,249	+
+ glycerol after 30 min	0.01 ± 0.006	1,683	-
+ glycerol & ALLN after 30 min	0.056 ± 0.03	7,899	+

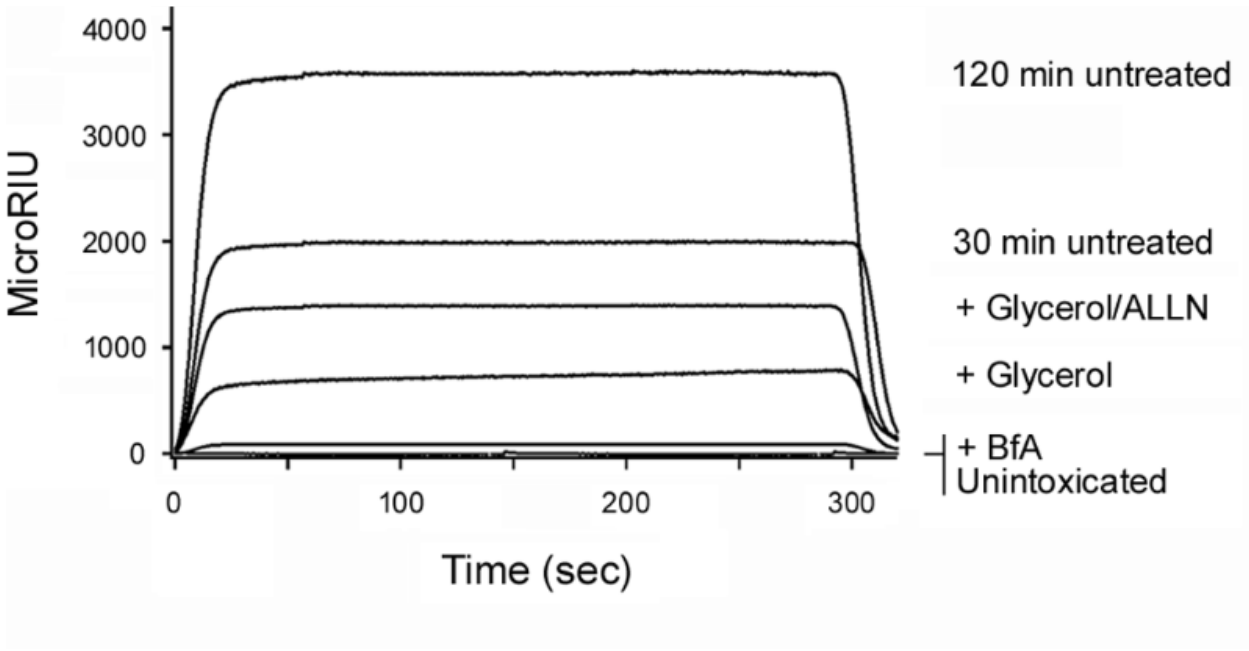

Figure 10. Glycerol prevents further translocation of CTA1 to the cytosol.

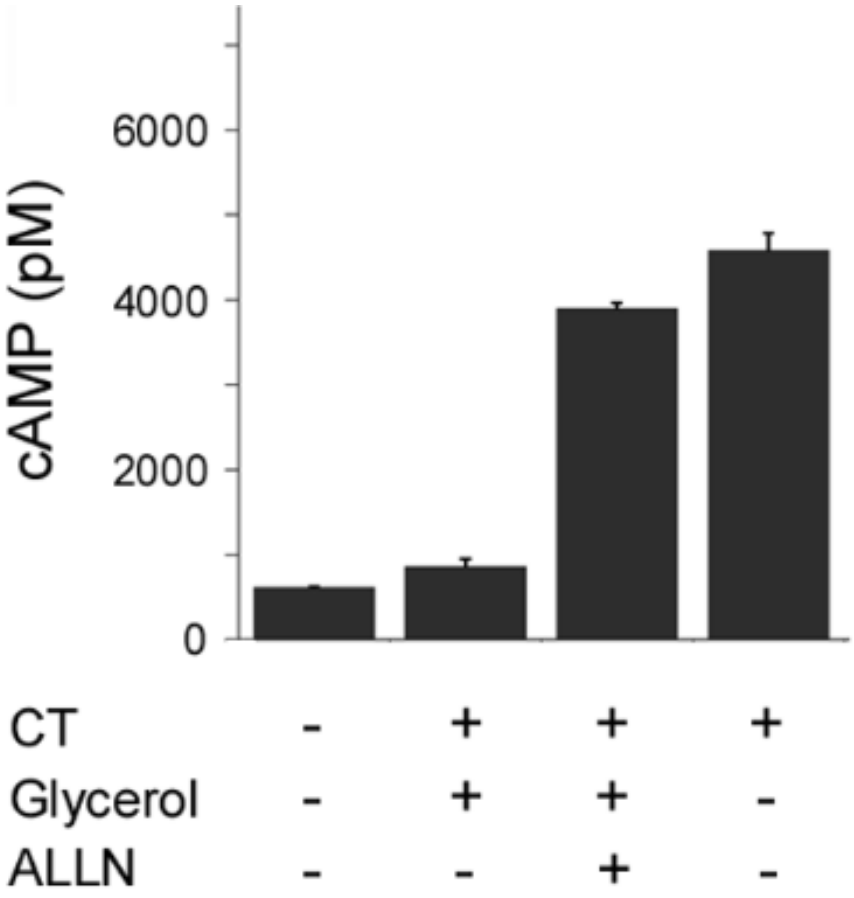

Figure 11. Glycerol prevents cAMP activation.

We have now established that a threshold quantity of cytosolic toxin must be reached in order to elicit a cytopathic effect, and CTA1 must be continually delivered to the cytosol in order to overcome the constant proteasome-mediated clearance of cytosolic toxin. We also have established the use of multiple drugs to block this delivery of CTA1 to the cytosol. We next looked into the possibility of recovery with the idea that post-exposure inhibition of toxin translocation could reverse the effects of intoxication. If a there is a threshold of CTA1 required to be present in the cytosol of a cell, there is the possibility of blocking translocation, after CTA1 has entered the cytosol and cAMP response to the intoxication has begun, allowing the cell to degrade the toxin already present and reverse the response initiated by that quantity of toxin. Table 4 shows data of a lengthier intoxication with and without treatment of glycerol after 1 hour of intoxication for an additional 2, 4, or 8 hours at 37°C. The quantity of cytosolic CTA1 and the levels of intracellular cAMP were recorded at each chase interval. Glycerol-treated cells contained less cytosolic CTA1 at 3, 5, and 9 hours of chase than untreated cells at the time of drug treatment.

Table 4. A post-exposure block of toxin translocation results in the time-dependent clearance of CTA1 from the cytosol.

Chase condition	ng cytosolic CTA1	CTA1 molecules per cell
No treatment		
1 hr	0.05 ± 0.01	7,164
3 hr	0.39 ± 0.17	60,110
5 hr	7.6 ± 3.8	1,169,661
9 hr	102 ± 27	15,712,156
+ glycerol after 1 hr		
3 hr	0.02 ± 0.007	3,264
5 hr	0.04 ± 0.02	6,602
9 hr	0.06 ± 0.03	9,390

CHAPTER FOUR: DISCUSSION

We have shown a correlation between cytosolic CTA1 quantity and toxin activity through breakthrough research techniques. The ability to determine the quantity of cytosolic CTA1 with the use of SPR and cytosolic CTA1 activity through the use of direct cAMP measurements makes this research pivotal to the future development of toxin research and post-exposure toxin therapeutics. We have for the first time directly calculated toxin levels in the cytosol of a host cell and the cellular response it elicits. This data supports the idea that there is a minimal quantity of CTA1 that must persist in the cytosol to induce the cAMP response and resulting toxicity to the cell. We have determined the threshold quantity of cytosolic CTA1 is required to elicit a toxic effect is.05ng. A kinetic requirement of greater than 30 minutes of toxin exposure also appears necessary for active intoxication.

CTA1 must be continually delivered from the cell surface to the cytosol in order to overcome the constant proteasome-mediated clearance of cytosolic toxin, and we believe this is direct evidence of the presence of this threshold requirement. A threshold model is contrary to the current idea that only one molecule of cytosolic toxin is required to affect host cells irreversibly. Using various drug treatments that block CTA1 translocation to the cytosol, we have documented a decrease in the amount of CTA1 that was in the cytosol prior to drug treatment due to degradation of CTA1 by the proteasome. Thus, proteasomal activity could lower the quantity of cytosolic CTA1 below the threshold when translocation to the cytosol is blocked even after toxin exposure. By blocking ER to cytosol translocation the cell is able to reduce toxin levels in the cytosol below the required threshold quantity. Treatment with glycerol at 1 hour post exposure and carried out to chase intervals outward to 9 hours support this possibility.

We have also shown decreased toxin activity with drug treatment that we attribute to regulatory factors native to the cell reducing and reversing toxin activity. This would refute the idea that only one molecule of cytosolic toxin is sufficient to generate a toxic effect. ADP-ribosylation of G proteins as well as cAMP levels are regulated events that can be reversed by cellular mechanisms. This implies it is possible to reverse these effects of intoxication and fully recover from intoxication. When CTA1 passage from the ER to the cytosol was blocked after exposure to the toxin, proteasomal activity lowered the amount of toxin already in the cytosol below the threshold quantity. This post-exposure block of toxin translocation will both allow the intoxicated cell to clear the cytosol of toxin and reverse the effects of intoxication.

Glycerol does not represent an administrable drug but is used for proof-of-principal. However, PBA is an FDA-approved drug used for disorders involving the urea cycle. GA is being used in clinical trials against cancer. PBA and GA block by different mechanisms. Due to the fact that they act on different sites of intoxication, we can use them together for an additive effect of preventing intoxication. In future work we will establish the therapeutic utility of these drugs using Caco-2 cells, a polarized intestinal epithelial cell line. We also will be adding much longer chase times, carried out to 48 hours, in efforts to monitor intoxication and the reversal of intoxication over the life-span of an intestinal epithelial cell *in vivo*. We will also establish the minimal effective dose of these drugs as they are a novel use for both PBA and GA. CTA1 translocation has not been previously targeted as a method for intoxication treatment or reversal, but our results indicate it to be a promising target.

We believe with this data we have shown cholera is a reversible process that could be treated after the onset of symptoms. The research of cholera specific entry will bring more light to research of AB toxins as a whole. There are many AB toxins such as diphtheria, pertussis, and

shiga toxin that cause a variety of life threatening diseases and have similarities amongst their cellular mechanisms. CT is secreted into the intestinal lumen, but moves in a retrograde manner through the vesicles of the cell to act within the cytosol of host cells, a common trend among AB toxins., Ricin, shiga toxin, pertussis toxin, and *Escherichia coli* type I heat-labile enterotoxin also move in this retrograde fashion from the cell surface and exploit ERAD to gain entry to the cytosol where there A subunits are functional. This makes research on cholera especially significant and relevant as a model for other AB toxin research and treatment. The threshold and reversible nature of CT intoxication may be present for other toxins. Research of cholera specific entry will bring more light to research of related toxin-mediated diseases as a whole.

CTA hijacks the normal cellular mechanism of ERAD, a quality control system in place to rid the cell of misfolded proteins. It is understood that using these drugs to inhibit ERAD in order to prevent CTA1's export to the cytosol may also interfere with the cell's necessity to export misfolded proteins for degradation. This may lead to cell toxicity directly. However Hsp90 and PBA are already administered therapeutics with low toxicity during the required short term treatment. Both drugs are also effective at relatively lower concentrations then what is normally used. The localized delivery of therapeutics directly to the intestine that does not require a systemic administration also aids in reducing side effects. In future work we hope to see over the longer time course of drug treatment, once cells have shown recovery from intoxication, no adverse side effects. The cells may be able to cope with certain steps in the ERAD system being suspended for certain periods of time. These experiments will be carried out once again in CaCo2 cells, a polarized epithelial cell line, to show the clinical relevance in a more cholera intoxication-specific cell type.

These experiments have stringently established a method for cholera toxin research. We have for the first time determined and precisely quantified the threshold quantity of cytosolic toxin that must be reached in order to elicit a cytopathic effect. Furthermore, the cytosolic pool of toxin must be maintained at this level for active intoxication. With this data we intend to further our understanding of cholera toxin's mechanism within the human body and be able to continue with more educated drug discovery concerning how cholera intoxication can be treated and reversed once a person is intoxicated. Our research suggests it is possible to both treat and reverse the effects of cholera even after the onset of disease, which opens the door to new therapeutic strategies.

LIST OF REFERENCES

1. WHO (June 2010). Cholera Fact sheet. WHO Cholera media centre. Retrieved July 11, 2011, from http://www.who.int/mediacentre/factsheets/fs107/en/
2. Sack DA, Sack RB, Nair GB, Siddique AK (2004) Cholera. Lancet 363: 223-233.
3. Mahajan V, Sajan SS, Sharma A, Kaur J.Ringers lactate vs Normal saline for children with acute diarrhea and severe dehydration- a double blind randomized controlled trial. Indian Pediatr. 2012 Dec;49(12):963-8. Epub 2012 Mar 30. Department of Pediatrics, Government Medical College and Hospital,Chandigarh, India
4. Taylor M, Navarro-Garcia F, Huerta J, Burress H, Massey S, et al. (2010) Hsp90 is required for transfer of the cholera toxin A1 subunit from the endoplasmic reticulum to the cytosol. J Biol Chem 285: 31261-31267.
5. Maya Kitaoka, Sarah T. Miyata, Daniel Unterweger and Stefan Pukatzki Antibiotic resistance mechanisms of Vibrio cholera J Med Microbiol April 2011 vol. 60 no. 4 397-407
6. Watson, P. and R.A. Spooner, Toxin entry and trafficking in mammalian cells. Adv Drug Deliv Rev, 2006. 58(15): 1581-1596.
7. Wernick NLB, Chinnapen DJ-F, Cho JA, Lencer WI (2010) Cholera toxin: an intracellular journey into the cytosol by way of the endoplasmic reticulum. Toxins 2: 310-325.
8. Spangler BD (1992) Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. Microbiol Rev 56: 622-647.

9. Orlandi PA, Curran PK, Fishman PH (1993) Brefeldin A blocks the response of cultured cells to cholera toxin. Implications for intracellular trafficking in toxin action. J Biol Chem 268: 12010-12016.

10. Guimaraes CP, Carette JE, Varadarajan M, Antos J, Popp MW, et al. (2011) Identification of host cell factors required for intoxication through use of modified cholera toxin. J Cell Biol 195: 751-764.

11. Lencer WI, de Almeida JB, Moe S, Stow JL, Ausiello DA, et al. (1993) Entry of cholera toxin into polarized human intestinal epithelial cells. Identification of an early brefeldin A sensitive event required for A1-peptide generation. J Clin Invest 92: 2941-2951.

12. Lencer WI, Delp C, Neutra MR, Madara JL (1992) Mechanism of cholera toxin action on a polarized human intestinal epithelial cell line: role of vesicular traffic. J Cell Biol 117: 1197-1209.

13. Shen Y, Hendershot LM. ERdj3, a stress-inducible endoplasmic reticulum DnaJ homologue, serves as a cofactor for BiP's interactions with unfolded substrates. Molecular biology of the cell. 2005;16(1):40-50.

14. Taylor M, Banerjee T, Navarro-Garcia F, Huerta J, Massey S, et al. (2011) A therapeutic chemical chaperone inhibits cholera intoxication and unfolding/translocation of the cholera toxin A1 subunit. PLoS ONE 6: e18825.

15. Rodighiero C, Tsai B, Rapoport TA, Lencer WI (2002) Role of ubiquitination in retro-translocation of cholera toxin and escape of cytosolic degradation. EMBO Rep 3: 1222-1227 Epub 2002 Nov 1221.

16. Bernardi KM, Forster ML, Lencer WI, Tsai B (2008) Derlin-1 facilitates the retro-translocation of cholera toxin. Mol Biol Cell 19: 877-884.

17. Romisch K. Surfing the Sec61 channel: bidirectional protein translocation across the ER membrane. Journal of cell science. 1999;112 (Pt 23):4185-91.

18. Bar-Nun S. The role of p97/Cdc48p in endoplasmic reticulum-associated degradation: from the immune system to yeast. Curr Top Microbiol Immunol. 2005;300:95-125.

19. Kothe M, Ye Y, Wagner JS, De Luca HE, Kern E, Rapoport TA, et al. Role of p97 AAA-ATPase in the retrotranslocation of the cholera toxin A1 chain, a non-ubiquitinated substrate. The Journal of biological chemistry. 2005;280(30):28127-32.

20. McConnell E, Lass A, Wojcik C. Ufd1-Npl4 is a negative regulator of cholera toxin

21. Giodini A, Cresswell P. Hsp90-mediated cytosolic refolding of exogenous proteins internalized by dendritic cells. The EMBO journal. 2008;27(1):201-11.

22. Schmitz A, Herrgen H, Winkeler A, Herzog V (2000) Cholera toxin is exported from microsomes by the Sec61p complex. J Cell Biol 148: 1203-1212.

23. Brodsky JL, McCracken AA. ER-associated and proteasomemediated protein degradation: how two topologically restricted events came together. Trends in cell biology. 1997;7(4):151-6.

24. M. Bajorek, M. H. Glickman Ubiquitin-proteasome system. Cellular and Molecular Life Sciences CMLS June 2004, Volume 61, Issue 13, pp 1579-1588

25. London E, Luongo CL. Domain-specific bias in arginine/lysine usage by protein toxins. Biochem Biophys Res Commun. 1989;160(1):333-9.

26. Hazes B, Read RJ (1997) Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. Biochemistry 36: 11051-11054.

27. Ampapathi RS, Creath AL, Lou DI, Craft JW, Jr., Blanke SR, et al. (2008) Order-disorder-order transitions mediate the activation of cholera toxin. J Mol Biol 377: 748-760.

28. De Haan L, Hirst TR (2004) Cholera toxin: a paradigm for multi-functional engagement of cellular mechanisms (Review). Mol Membr Biol 21: 77-92.

29. Kato, J., J. Zhu, C. Liu and J. Moss, Enhanced sensitivity to cholera toxin in ADP-ribosylarginine hydrolase-deficient mice. Mol Cell Biol, 2007. 27(15): 5534-5543

30. Chang, F.H. and H.R. Bourne, Cholera toxin induces cAMP-independent degradation of Gs. Biol Chem, 1989. 264(10): 5352-5357.

31. Yamaizumi M, Mekada E, Uchida T, Okada Y (1978) One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. Cell 15: 245-250.

32. Yoshio Okada et al. One molecule of Diptheria Toxin fragment A Introduced into a cell can kill the cell. Cell, September 1978. Volume 15, 245-250.

33. Massey S, Banerjee T, Pande AH, Taylor M, Tatulian SA, et al. (2009) Stabilization of the tertiary structure of the cholera toxin A1 subunit inhibits toxin dislocation and cellular intoxication. J Mol Biol 393: 1083-1096.

34. Banerjee T, Pande A, Jobling MG, Taylor M, Massey S, et al. (2010) Contribution of subdomain structure to the thermal stability of the cholera toxin A1 subunit. Biochemistry 49: 8839-8846.

35. Taylor M, Banerjee T, VanBennekom N, Teter K (2012) Detection of toxin translocation into the host cytosol by surface plasmon resonance. Journal of Visualized Experiments 59: doi: 10.3791/3686.