ARTICLE
Journal of Cellular Biochemistry 105:129–135 (2008)

Journal of Cellular Biochemistry

Un-Nicked BoNT/B Activity in Human SHSY-5Y Neuronal Cells

Xuerong Shi,* Gregory E. Garcia, Madhusoodana P. Nambiar, and Richard K. Gordon

Department of Biochemical Pharmacology, Division of Biochemistry, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, Maryland 20910-7500

ABSTRACT

BoNT/B holotoxin (HT) from the native source is a mixture of nicked and un-nicked forms. A previous study showed that while un-nicked HT could be transcytosed by intestinal epithelial cells, they did not correlate this with proteolytic activity or biological effect(s). Un-nicked HT is likely to be present in BoNT biological warfare agents (BWA), so it is important to investigate the relative toxicity of un-nicked HT in this BWA. To address this issue, we purified un-nicked HT from commercial sources and evaluated its ability to cleave substrates both in vitro and in vivo, and its effects on vesicle trafficking. The un-nicked HT was unable to cleave VAMPTide^{TM} substrate used for in vitro proteolytic assays. Brief digestion of the un-nicked toxin with trypsin resulted in significant activation of the toxin proteolytic ability. SHSY-5Y human neuroblastoma cells were used to examine HT uptake and activation in vivo. Vesicle trafficking can be measured following K^+ stimulation of cells preloaded with [^{3}H]-noradrenaline (NA). We found that highly purified un-nicked HT did inhibit NA release but at much reduced levels compared to the nicked toxin. That the reduction in NA release was due to BoNT effects on SNARE proteins was supported by the finding that VAMP-2 protein levels in un-nicked toxin treated cells was greater than those treated with nicked toxin. These results demonstrate that although un-nicked HT has markedly reduced toxicity than the nicked form, due to the preponderance in BoNT/B preparations from the native bacteria, it is a major source of toxicity. J. Cell. Biochem. 105: 129–135, 2008.

KEY WORDS: BOTULINUM NEUROTOXIN; SYNAPTOBREVIN-2; BACTERIAL TOXIN; SYNAPSE; VESICLE CELLULAR TRAFFICKING; ENDOCYTOSIS; PROTEASOME

The family of botulinum neurotoxins (BoNT) is one of the most potent toxins in nature [Gill, 1982]. All the members or serotypes of the family have two distinct components; the larger, heavy chain fragment (100 kDa), is required for binding and translocation of the toxin specifically into neuronal cells, while the smaller, light chain fragment (50 kDa), contains the enzymatic portion responsible for the toxicity observed with botulinum intoxication [Bandyopadhyay et al., 1987; Montecucco and Schiavo, 1994; Deloye et al., 1997; Pellizzari et al., 1999]. Both light and heavy chains in all the family members are held together by a disulfide bond [Simpson, 1979; DasGupta, 1990; Humeau et al., 2000], which is required for toxins to have their toxicity against neuronal cells [Schiavo et al., 1990; Simpson et al., 2004; Fischer and Montal, 2007a]. The light chain is translocated into the cells through a pore or channel formed by the heavy chain [Koriazova and Montal, 2003; Fischer and Montal, 2007b]. The physical separation of light chain from heavy chain is required for Zn^{2+}-dependent light chain (LC) endopeptidase activity inside cells, similarly as for the in vitro proteolytic activity [Stecher et al., 1989]. Cleavage of a SNARE protein by LC endopeptidase results in inhibition of release of the neurotransmitter, acetylcholine, from the synaptic vesicles, leading to muscular flaccid paralysis [Schiavo et al., 2000; Simpson, 2004]. The different members of the BoNT family cleave different SNARE proteins. BoNT/A, C and E cleave

Abbreviation used: BoNT, botulinum neurotoxin; BWA, biological warfare agents; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer; HPLC, high pressure liquid chromatography; HT, holotoxin; NA, noradrenaline; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; TCEP, tris-(2-carboxyethyl)-phosphine hydrochloride; TMB, tetramethyl benzidine; VAMP-2, vesicle-associated membrane protein-2/synaptobrevin-2.

Grant sponsor: Defense Threat Reduction Agency (DTRA); Grant number: #3.10017_06_WR_B.

*Correspondence to: Xuerong Shi, Department of Biochemical Pharmacology, Division of Biochemistry, Walter Reed Army Institute of Research, 503 Robert Grant Avenue, Silver Spring, MD 20910-7500.
E-mail: xuerong.shi@amedd.army.mil

Received 21 December 2007; Accepted 2 April 2008 • DOI 10.1002/jcb.21800 • 2008 Wiley-Liss, Inc.
Published online 5 May 2008 in Wiley InterScience (www.interscience.wiley.com).

SNAP-25, while BoNT/B, D, F and G cleave synaptobrevin-2/VAMP-2. Unlike the other serotypes, BoNT/C can also cleave syntaxin [Barr et al., 2005].

BoNT/B holotoxin (HT) purified from the native source, *Clostridium botulinum* bacteria, is a mixture of nicked and un-nicked forms [Sathyamoorthy and DasGupta, 1985], and the ratio of un-nicked to nicked was usually around 2:1 [Shone and Roberts, 1994]. The un-nicked form of BoNT/B toxin, either reduced or non-reduced, was reported to penetrate into neuronal cells faster than the nicked form [Maksymowych and Simpson, 2004], but its proteolytic activity was not determined.

In this present study, we tested the proteolytic ability and cellular effects of un-nicked BoNT/B HT. The un-nicked form of the HT was purified from a commercial source by HPLC size exclusion chromatography under SH-reducing conditions. The proteolytic activity was measured by using a cell-free peptide fluorescence resonance energy transfer (FRET) assay. The toxin ability to affect cellular activity was examined by a [^{3}H]-noradrenaline (NA) release assay and measurements of VAMP-2 SNARE levels in the human neuroblastoma cell line SHSY-5Y. Our results demonstrate that the purified un-nicked form of BoNT/B was not active in vitro, but *does* have a modest in vivo activity.

MATERIALS AND METHODS

CELL LINE

SHSY-5Y, a human neuroblastoma cell line, was purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in a 95% air and 5% CO_2 humidified incubator at 37°C. Typically, 2×10^5 cells were seeded in 12-well plate, while 2×10^6 cells were seeded in 10 cm plates in various experiments. Cell monolayers were ~70% confluent when they were treated with BoNT/B toxin.

UN-NICKED BoNT/B TOXIN PURIFICATION BY HPLC

BoNT/B HT isolated from the native source was purchased from BBTech Inc. (Dartmouth, MA). The toxin was reduced by treatment with 1 mM TCEP for 30 min at 24°C prior to purification by size-exclusion chromatography over an HPLC Protein PAK SW300 7.5 mm × 30 cm column (Waters, Corp). The HPLC consisted of a Beckman 125 pumpset, 508 autosampler, and 168 PDA controlled by Beckman 32 Karat Ver. 5.0 software. Fractionated proteins were collected using a Gilson F240 fraction collector. Proteins were eluted at a flow rate of 0.41 ml/min in running buffer consisting of 0.15 M NaCl, 0.1 M phosphate buffer (pH 6.8) and 10 mM DTT. The absorbance at 215 and 280 nm were monitored. The column was calibrated by injection of individual standard proteins, or standard protein mixture, and the elution volume (V_e) determined for each protein. The protein standards used were the followings: carbonic anhydrase (29 kDa), bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), appoferritin (443 kDa), and thyroglobulin (669 kDa) (all from the Cat.# MW-GF-1000 protein MW marker kit; Sigma-Aldrich Co.), prepared as described by the manufacturer. The void volume (V_o) was determined by chromatography of blue-dextrin. The elution volume fraction (V_e/V_o) was calculated and plotted versus the respective protein Log (MW) to derive a standard curve by linear regression analysis. Pooled fractions of the un-nicked BoNT were concentrated by ultrafiltration through Vivaspin 500 (Vivascience, AG) concentrators with 10 kDa MWCO (molecular weight cut off) in a Beckman refrigerated fixed angle rotor microfuge at 4°C as per manufacturers instructions.

BoNT/B LIGHT CHAIN FRET VAMPTide™ ASSAY

HPLC-purified un-nicked BoNT/B HT was incubated with 30 μg/ml trypsin in 0.1 M sodium phosphate (PH 7.5) at 24°C for various time periods. The trypsin activity was then inhibited with 0.9 mg/ml trypsin soybean inhibitor.

The activity of BoNT/B LC endopeptidase was measured using a modified FRET VAMPTide™ assay as described by List Biological Laboratories (Campbell, CA). Briefly, 0.5 μg BoNT/B HT in 10 μl of 0.1 M sodium phosphate buffer was added to wells containing 138.5 μl of 50 mM HEPES (pH 7.5) with 0.05% (v/v) Tween 20, 5 mM DTT, and 0.25 mM $ZnCl_2$ in a 96-well Costar plate (Corning Life Science, Corning, NY). As a positive control, recombinant BoNT/B LC (MetaBiologics, Madison, WI) was added to give a final concentration of 6 nM. The plates were sealed with a plastic sealer and incubated at 37°C for 15 min, and 1.5 μl of 200 μM VAMPTide™ substrate was then added to start the reaction. The plates were mixed for 30 s by the plate reader, and then immediately read using the kinetic program of a Tecan model Microplate Reader (Tecan Systems, San Jose, CA). The wells were read for 60 cycles each per 30 s at an emission wavelength of 415 nm and an excitation wavelength of 300 nm. The proteolytic activity of BoNT LC endopeptidase was represented as the slope of the linear portion of the kinetic curve determined with GraphPad Prism Ver. 4.1 software (GraphPad Software, San Diego, CA).

[^{3}H]-NA RELEASE ASSAY

The release of [^{3}H]-NA was determined by using the method previously described by Murphy et al. [1991] with minor modifications. Briefly, SHSY-5Y cultures were grown in 12-well plates to 70% confluency using RPMI 1640 media. Cultures were incubated with BoNT/B HT for 48 h. Untreated control cultures were also prepared. The cells were washed twice with HBS buffer (HEPES buffer saline) (135 mM NaCl, 5 mM KCl, 0.6 mM $MgSO_4 \cdot 7H_2O$, 2.5 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, 6 mM D-glucose (dextrose), 0.2 mM ascorbic acid, 0.2 mM pargyline). Cells were labeled by incubation with HBS containing 50 nM [^{3}H]-NA (10.9 Ci/mmol, PerkinElmer). After 1 h incubation, the isotope-supplemented HBS was removed from the cells, and the cells were washed four times with HBS. The cells were then evoked to release [^{3}H]-NA by stimulation with 100 mM K^+ for 5 min at 37°C. Following the stimulation, the cells were washed twice with HBS. Unreleased [^{3}H]-NA was extracted from the cells with 0.1% Triton X-100 (1 ml per well). 0.5 ml of cell lysate was added to 3 ml scintillation cocktail (Ultima Gold, PerkinElmer Inc., Waltham, MA), and the radioactivity was measured with a 1450 LSC & Luminescence counter (PerkinElmer Inc.). The level of [^{3}H]-NA

released on stimulation was determined as the difference of [^{3}H]-NA remaining in cells after K^+ exposure expressed as a percentage of unstimulated control cells.

VAMP-2 ELISA ASSAY

The effect of BoNT/B on its specific intracellular substrate, VAMP-2, was measured in the human neuoroblastoma cell line SHSY-5Y. Cleavage of VAMP-2 by BoNT/B LC endopeptidase was quantified by a modified sandwich ELISA previously described by Honer et al. [2002]. SHSY-5Y cell cultures were prepared in 10 cm plates, and treated with BoNT/B HT (10 μg/ml) in RPMI 1640 culture medium for 48 h. After treatment, the cells were lysed in radio-immunoprecipitation assay (RIPA) buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% glycerol, and 1% Triton X-100, supplemented just-before-use with freshly prepared phosphatase and protease inhibitors (1 mM orthovanandate, 10 mM NaF, 10 μg/ml leupeptin, 25 μg/ml aprotinin and 50 μg/ml PMSF). BoNT-untreated control cultures were prepared and processed similarly. The ELISA was performed in 96-well microtiter plates coated with 1–2 μg/ml goat anti-human VAMP-2 antibody (Proteintech, Chicago, IL) at 4°C overnight. Lysates from cell cultures were added to antibody-coated wells and incubated at room temperature for 2 h. After several washes with 0.5% Tween-20 in PBS, a 1:1,000 diluted anti-mouse antibody conjugated with horseradish peroxidase (Cell Signaling, Danvers, MA) was added in a volume of 0.1 ml, and incubated at room temperature for an additional 1 h after a 1:1000 diluted anti-human VAMP-2 antibody (QED Bioscience, San Diego, CA) was added in a volume of 0.1 ml, and incubated at room temperature for 1 h. Immunoreactivity was measured following addition of the tetramethyl benidine (TMB) reagent color reaction (KPL Inc., Gaithersburg, MD) by development for 30 min. The plate was read in a Spectramax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA) under control of the Spectramax software Ver. 5.1. The data was analyzed using Microsoft Excel. Protein levels for the cell lysate samples were determined using a Bradford Protein Assay kit (Bio-Rad Inc., Hercules, CA). The ELISA plate results were normalized to protein content.

SDS–PAGE METHODS

BoNT toxin proteins were analyzed by SDS–PAGE as described by Schägger and von Jagow [1987]. Briefly, 500 ng of BoNT toxin proteins were prepared in a non-reducing loading buffer, and then denatured by heating at 95°C in a water both for 7 min. The samples were then fractionated by electrophoresis through 10% non-reducing gels (Pre Cast PAGE gels, Life Therapeutics, Clarkston, GA) in a Bio-Rad Mini-protean3 Electrophoresis Module. Proteins were visualized by silver-staining utilizing the SilverSNAP Stain kit II (Pierce Biotechnology, Rockford, IL) following manufacturer's instructions. Densitometry images of the stained gels were captured with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). Protein bands were quantified using ImageQuant software Ver. 5.1 (Molecular Dynamics, CA).

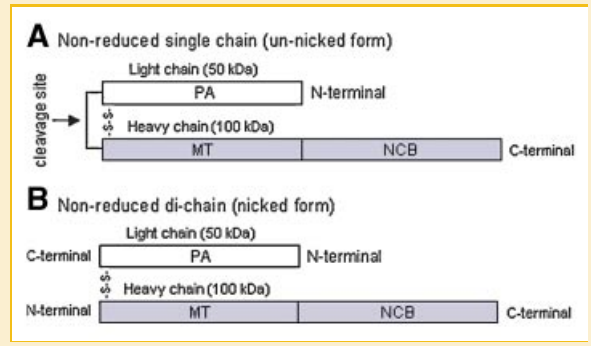

Fig. 1. Schematic diagram of the structures of the un-nicked and nicked BoNT/B HT. The proteolytic activity (PA), membrane translocating (MT), and neuronal cell binding domains (NCB) are indicated. The location of the disulfide bond is shown as S-S and the activating cleavage site is shown with the arrow.

RESULTS

UN-NICKED BoNT/B HOLOTOXIN PURIFICATION WITH HPLC SIZE EXCLUSION CHROMATOGRAPHY

BoNT/B HT purified from its natural source (*C. botulinum*) consists of two different forms, the nicked (di-chain), and the un-nicked (single chain), with both forms having the same molecular size (150 kDa, see Fig. 1). To isolate the un-nicked form from the nicked form, we first treated the toxin with a disulfide bond reducer, tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP). TCEP was able to dissociate light and heavy chains in the nicked form of the toxin by breaking the inter-disulfide bond that holds the two chains together, and was found to maximally reduce the HT at a concentration of 1 mM (Shi et al., manuscript in preparation). TCEP did not dissociate the un-nicked or single chain form of the toxin even though it could break its intra-disulfide bond. The reduced BoNT/B HT was then fractionated by size-exclusion HPLC. Chromatography of the purified un-nicked HT is shown in Figure 2A. Purified reduced un-nicked chain was compared with the reduced nicked form in this system as shown in Figure 2B. Reduced un-nicked BoNT/B HT (solid line) sample yielded only one peak corresponding to 150 kDa, while reduced nicked BoNT/B HT (dotted line) sample yielded two peaks corresponding to ~50 kDa and 100 kDa, respectively, indicating validity of isolation and purification of the un-nicked BoNT/B HT.

To confirm the purity of the HPLC-processed un-nicked BoNT/B HT, the peak fraction was examined by SDS–PAGE. The purified un-nicked HT was compared to commercial BoNT/B HT that was treated with or without TCEP. Silver staining was used to make the protein detection more sensitive than Coomassie blue staining by a factor of 10 to 100. The HPLC purified un-nicked BoNT/B HT exhibited only one band equivalent to 150 kDa, while the commercial BoNT/B HT exhibited two more bands corresponding to the LC and HC in addition to the un-nicked chain (Fig. 3).

TRYPSIN TREATMENT ACTIVATES THE PROTEOLYTIC ACTIVITY OF HPLC-PURIFIED UN-NICKED BoNT/B HOLOTOXIN

The HPLC purified un-nicked BoNT/B HT was not active in the proteolytic FRET VAMPTide™ assay in conditions under which the

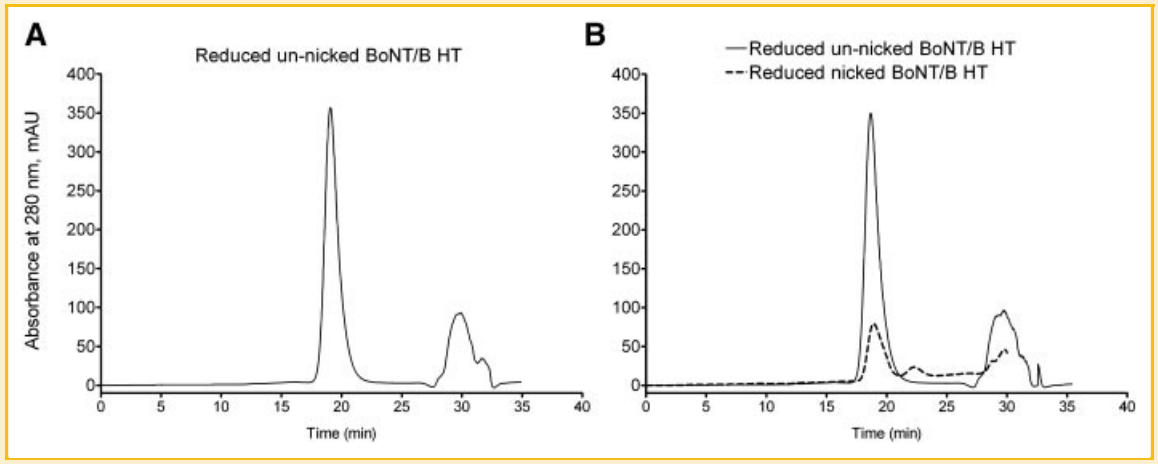

Fig. 2. Purification of un-nicked BoNT/B holotoxin by size-exclusion HPLC. A: Purified un-nicked BoNT/B HT. B: Comparison of reduced un-nicked (solid tracing) and nicked (dashed tracing) BoNT/B HT.

recombinant BoNT/B LC was active (Fig. 4A). Trypsin, an enzyme to digest proteins [Le Moine et al., 1994], digests BoNT/B toxins at pH 6.0 and results in the cleavage of the HT into two fragments with apparent molecular weight of 112 and 57 kDa fragments [Ohishi and Sakaguchi, 1977], and activation of the HT proteolytic activity. To determine whether trypsin digestion could activate the proteolytic ability of the HPLC-purified un-nicked BoNT/B HT by converting it into the nicked form, we treated the purified un-nicked HT with 30 μg/ml trypsin for different times: 0, 5, 10, 20, 40, and 60 min, respectively, at 24°C, and then measured its proteolytic activity in the FRET assay. We found that trypsin treatment converted the inactive HT to an active form in a time-dependent manner and the optimal digestion time was found to be 20 min under these conditions (Fig. 4B). This data demonstrates that the un-nicked BoNT/B HT has the ability to digest VAMP-2 if the HT is first converted to a nicked form.

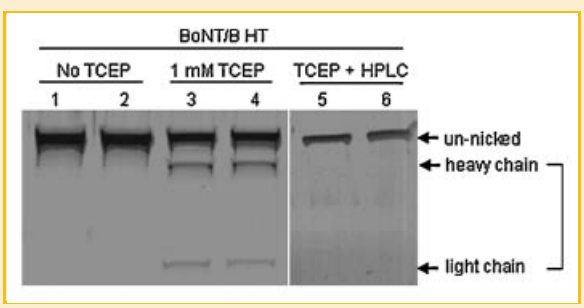

Fig. 3. SDS–PAGE analysis of various BoNT/B HT preparations. Lanes 1 and 2, unreduced commercial BoNT/B; lanes 3 and 4, commercial BoNT/B HT reduced with 1 mM TCEP for 30 min; lanes 5 and 6, purified un-nicked BoNT/B HT reduced similarly as samples prepared for lanes 3 and 4. SDS–PAGE in this experiment was a non-reducing gel, and lanes 1 and 2, 3 and 4, and 5 and 6 are two independent samples from individual experiments.

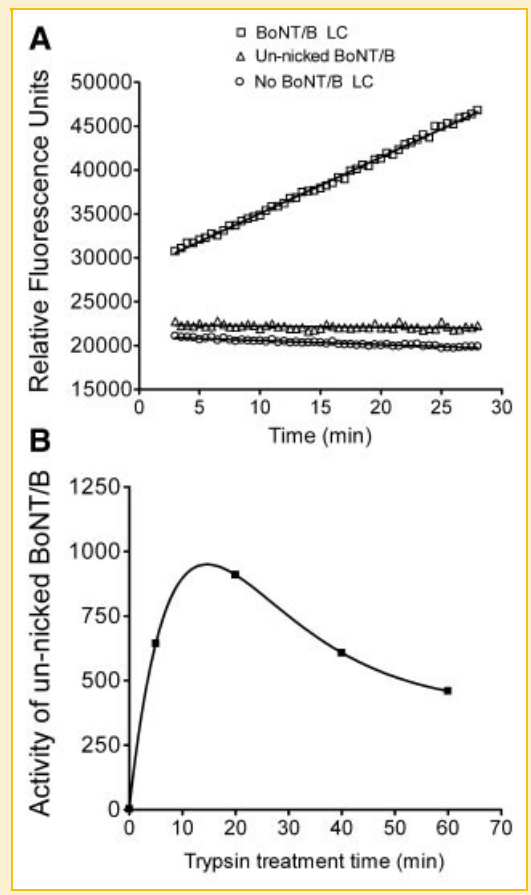

Fig. 4. Activation of purified un-nicked BoNT/B HT proteolytic activity by trypsin. A: Comparison of purified un-nicked BoNT/B HT to BoNT/B LC; 450 ng of purified un-nicked HT was reduced prior to the assay with TCEP (1 mM), 45 ng recombinant BoNT/B light chain was used as positive control. B: Time-dependency of trypsin-mediated activation of un-nicked BoNT/B HT peptidase. The activity of the HT was represented as a mean reaction rate (the slope of a linear line in A) and the effect of trypsin digestion over time (data not shown).

UN-NICKED BoNT/B HOLOTOXIN POORLY INHIBITS [^{3}H]-NA RELEASE IN HUMAN NEURONAL CELLS

Similar to in vitro activation by trypsin, the un-nicked BoNT/B HT may be activated by proteolytic enzymes and disulfide bond reducers on penetration of target cells since it is known that intracellular proteases, disulfide isomerases, and reductants exist in cells [Greene and Brophy, 1995; Biswas et al., 2006; Wang et al., 2008]. If so, this could result in the inhibition of SNARE vesicle trafficking by the originally un-nicked form of the toxin. To examine this hypothesis, we treated SHSY-5Y cells with different concentrations of purified un-nicked BoNT/B HT (2.5, 5.0, and 10.0 μg/ml) and then measured the effect on [^{3}H]-NA release. We found that un-nicked BoNT/B HT *does* possess the ability to inhibit [^{3}H]-NA release from these cells, albeit poorly, giving 0%, 14%, and 30% reduction of [^{3}H]-NA release at the tested concentrations. This is in contrast to the 86% inhibition seen from cells treated with the normal preparation used at 5 μg/ml (Fig. 5). SDS–PAGE as well as protein silver staining analyses showed that the ratio of un-nicked to nicked form in this preparation of BoNT/B HT was approximately 1.8:1 (gel data not shown). These data indicate that the un-nicked BoNT/B HT is able to impair function of neuronal cells, but its impact is much less than a mixture of nicked and un-nicked forms found in natural preparations.

UN-NICKED BoNT/B HOLOTOXIN WEAKLY CLEAVES VAMP-2 PROTEIN IN HUMAN NEURONAL CELLS

The observations described above suggest that the inhibition of cellular function is due to intracellular synaptic vesicle protein VAMP-2 cleavage by activation of un-nicked BoNT/B HT to an active form. To test this hypothesis, we treated SHSY-5Y cells with HPLC purified un-nicked BoNT/B HT. Control cultures were treated with the unprocessed preparation. After treatment, cell lysates were examined for intact VAMP-2. The VAMP-2 protein levels were measured using a capture ELISA assay. The results showed that

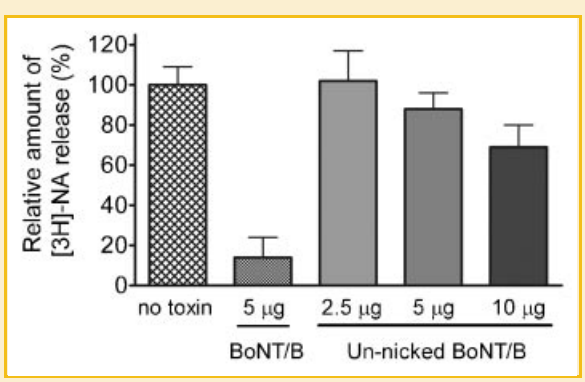

Fig. 5. Effect of BoNT/B preparations on the release of noradrenalin from SHSY-5Y cells. The percentage of [^{3}H]-NA released after K^+ stimulation was calculated and compared to the total radioactivity in the cells prior to stimulation. [^{3}H]-NA released from control cells without treatment was set to 100%. Data shown were obtained in duplicate wells and are the means ± SD of three experiments.

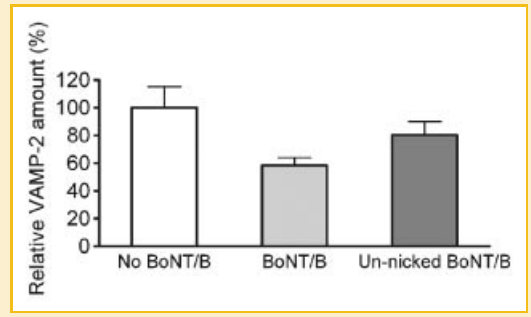

Fig. 6. Comparison of the in vivo activity of purified un-nicked BoNT and BoNT/B HT to cleave VAMP-2 protein in SHSY-5Y cells. A capture ELISA was used to measure VAMP-2 in whole cell lysates following 48 h BoNT treatment. The 100% level of VAMP-2 in the cell lysate was determined from BoNT untreated cells. Data shown were obtained in triplicate wells and are the means ± SD of three experiments.

approximately 42% VAMP-2 protein was cleaved by the unprocessed BoNT/B compared to toxin untreated control cells. This is in contrast to the finding of 19% VAMP-2 protein cleaved by un-nicked BoNT/B toxin used at a similar concentration as the unprocessed preparation (Fig. 6). These data are consistent with those obtained from the [^{3}H]-NA release assay, supporting the hypothesis that the un-nicked BoNT/B HT is marginally activated on uptake into these cells, which is reflected in their low proteolytic activity.

DISCUSSION

A large number of studies on BoNT/B HT report on the nicked HT [reviewed by Chaddock and Marks, 2006], but none so far have examined the role and fate of the un-nicked form. Since un-nicked HT could be a major component of the toxin in biological warfare preparations, it should be evaluated.

Previous studies have shown that enzymatic nicking of BoNT/B HT isolated from the native source increased the concentration of the nicked form as well as the proteolytic activity of the LC endopeptidase [Sathyamoorthy and DasGupta, 1985; Maisey et al., 1988; Hallis et al., 1996]. The extent of the increases of nicked forms and proteolytic activity were similar, and therefore it was assumed that the un-nicked form of BoNT/B HT had little or no proteolytic activity compared to the nicked form [Shone and Roberts, 1994]. Our observations support and quantify this assumption as highly purified un-nicked BoNT/B HT does not exhibit endopeptidase activity for VAMP-2 in vitro. This toxin preparation, however, became highly active following trypsin treatment, indicating that the toxin preparation maintained the intrinsic proteolytic activity. But the question remained is the un-nicked form active in vivo.

To answer this question, we incubated highly purified un-nicked toxin with a BoNT susceptible human neuroblastoma cell line SHSY-5Y. We examined cellular function by measuring the release of [^{3}H]-NA from these cells after 48 h incubation with the toxin. We found that there was a modest inhibition of about 19% (at the concentration of 5 μg) of the response relative to non-toxin treated

cells. This result suggests that the un-nicked toxin is taken up by the cells and activated intracellularly by some (as yet unidentified) protease(s) and reductants as there was no demonstrateable activation of the un-nicked BoNT/B HT activity following incubation of HT with the SHSY-5Y serum-containing medium or spent medium of SHSY-5Y cells (data not shown).

Purified un-nicked BoNT/B HT following trypsin digestion showed a comparable in vitro activity as that seen with a similar amount of nicked BoNT/B HT (data not shown). In contrast, the purified un-nicked form exhibited much less activity in the [^{3}H]-NA release assay in SHSY-5Y cells than standard BoNT/B preparations (containing a mixture of nicked and un-nicked BoNT) produced from native sources. The unpurified toxin mixture had a fivefold greater activity for blocking release of [^{3}H]-NA and exhibited a stronger activity for in vitro cleavage of VAMP-2 than the purified un-nicked toxin. The reduced activity of the un-nicked BoNT/B HT in vivo may be due to the persistence of uncleaved toxin or toxin losses from non-productive proteolytic cleavages.

Commercial preparations of BoNT/B HT are usually mixtures of nicked and un-nicked forms. In this report we demonstrate that HPLC-purified un-nicked BoNT/B HT does not have activity in vitro but it *does* have modest activity in vivo. Based on our findings, the relative contributions of the nicked and un-nicked forms for the in vivo activity of the preparation are 39%/μg for the nicked, and 9%/μg for the un-nicked as shown in Table I. For the toxin preparation used in this study, given the ratio of un-nicked to nicked form in our commercial BoNT/B HT preparation was 1.8:1, 71% of the in vivo activity was due to the nicked component, and 29% from the un-nicked component. The active form derived from the un-nicked component may ultimately depend on intracellular proteolysis to generate a 'nicked' active form, and this may be an inefficient process, but there may be other contributing factors such as un-nicked uptake efficiency and cellular stability.

The biological significance of un-nicked botulinum toxin B compared to nicked botulinum toxin B is that first, the un-nicked toxin is much less toxic to SHSY-5Y cells compared to nicked toxin. Second, by virtue of its observed toxicity in vivo but not in vitro (Fig. 4), we have for the first time demonstrated that separation of the heavy chain from the light chain prior to entering the cell is not necessary for BoNT intoxication, although the efficiency is decreased compared to nicked toxin. These results suggest that prophylactic and therapeutic strategies for BoNT intoxication will have to take into account that both forms may be present. As such, a binding intervention strategy would be useful to prevent uptake of both nicked and un-nicked forms. This intervention might be an antibody to the HC. Therapies with reducing agents to separate the two chains—the light from heavy, would not be effective for the un-nicked toxin; unlike the nicked form which could be rendered inactive by an agent that reduces the disulfide bond (Fig. 1) such that the light chain no longer has the heavy chain to direct it into the cell. In the case where the toxins enter the cell, the un-nicked form is much less toxic than the nicked form, suggesting that either toxin translocation across the membranes or the cellular process of nicking the toxin is not efficient. Nevertheless, a cell delivered therapeutic for botulinum intoxication would be identical for both forms of the toxin targeting the light chain proteolytic activity. To date, however, there are neither delivery systems to target neuronal cells with therapeutics nor any high potency small molecule inhibitors of the proteolytic activity of the light chain. The light chain must be separated from the heavy chain to exhibit cleavage of VAMP (in vitro results, Fig. 4); as our cellular studies demonstrate that the potent toxicity of nicked toxin and reduced toxicity in the cells of un-nicked toxin for both noradrenaline release and VAMP cleavage (Figs. 5 and 6, respectively). Since intracellular toxin is long lasting, indeed more than 3 months for BoNT/A and several weeks for BoNT/B [Sloop et al., 1997; Eleopra et al., 1998; de Paiva et al., 1999; Jurasinski et al., 2001], it is unlikely that VAMP-2 replacement would overcome BoNT/B toxicity in the neuronal cell unless the VAMP-2 were continuously replaced over the time frame of BoNT/B presence. How BoNT toxin avoids clearance from the cell is not yet clear. It may be possible to modulate ubiquitin ligases to recognize the toxin and increase its removal rate. Taken together, our data suggests that intracellular therapeutics will be effective for both forms of the toxin, nicked and un-nicked. In contrast, therapeutics against the toxin before it enters the neuronal cell will likely require different compounds, such as antibodies to prevent binding, and adjunct treatments to target the disulfide bond in the nicked form.

TABLE I. Comparison of Activities of Un-Nicked and Nicked BoNT/B HT

	No BoNT HT Treatment	Commercial BoNT/B HT (un-nicked and nicked)[a]	Purified Un-nicked BoNT/B HT
Cleavage of VAMP-2	0[b]	42[b]	19[b]
Total toxin used (μg)	0	5	5
Un-nicked toxin (μg)	0	3.2[c]	5
Nicked toxin (μg)	0	1.8[d]	0
Activity from un-nicked (%)	N/A	29[e]	100
Activity from nicked (%)	N/A	71[f]	0
Activity from un-nicked (%/μg)	N/A	9[g]	N/A
Activity from nicked (%/μg)	N/A	39[h]	N/A

N/A = not applicable.
[a]Ratio of un-nicked to nicked is 1.8:1.
[b]Data from Figure 6.
[c]$5 \times 1.8/(1.8 + 1) = 3.2$.
[d]$5 - 3.2 = 1.8$.
[e]$19 \times 3.2/5/42 = 29$.
[f]$100 - 29 = 71$.
[g]$29/3.2 = 9$.
[h]$71/1.8 = 39$.

ACKNOWLEDGMENTS

This material has been reviewed by the Walter Reed Army Institute of Research and there is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense.

REFERENCES

Bandyopadhyay S, Clark AW, DasGupta BR, Sathyamoorthy V. 1987. Role of the heavy and light chains of botulinum neurotoxin in neuromuscular paralysis. J Biol Chem 262(6):2660–2663.

Barr JR, Moura H, Boyer AE, Woolfitt AR, Kalb SR, Pavlopoulos A, McWilliams LG, Schmidt JG, Martinez RA, Ashley DL. 2005. Botulinum neurotoxin detection and differentiation by mass spectrometry. Emerg Infect Dis 11(10): 1578–1583.

Biswas S, Chida AS, Rahman I. 2006. Redox modifications of protein-thiols: Emerging roles in cell signaling. Biochem Pharmacol 71(5):551–564.

Chaddock JA, Marks PM. 2006. Clostridial neurotoxins: Structure–function led design of new therapeutics. Cell Mol Life Sci 63(5):540–551.

DasGupta BR. 1990. Structure and biological activity of botulinum neurotoxin. J Physiol (Paris) 84(3):220–228.

Deloye F, Doussau F, Poulain B. 1997. Action mechanisms of botulinum neurotoxins and tetanus neurotoxins. C R Seances Soc Biol Fil 191(3):433–450.

de Paiva A, Meunier FA, Molgó J, Aoki KR, Dolly JO. 1999. Functional repair of motor endplates after botulinum neurotoxin type A poisoning: Biphasic switch of synaptic activity between nerve sprouts and their parent terminals. Proc Natl Acad Sci USA 96(6):3200–3205.

Eleopra R, Tugnoli V, Rossetto O, De Grandis D, Montecucco C. 1998. Different time courses of recovery after poisoning with botulinum neurotoxin serotypes A and E in humans. Neurosci Lett 256(3):135–138.

Fischer A, Montal M. 2007a. Crucial role of the disulfide bridge between botulinum neurotoxin light and heavy chains in protease translocation across membranes. J Biol Chem 282(40):29604–29611.

Fischer A, Montal M. 2007b. Single molecule detection of intermediates during botulinum neurotoxin translocation across membranes. Proc Natl Acad Sci USA 104(25):10447–10452.

Gill DM. 1982. Bacterial toxins: A table of lethal amounts. Microbiol Rev 46(1):86–94.

Greene JJ, Brophy CI. 1995. Induction of protein disulfide isomerase during proliferation arrest and differentiation of SH5Y neuroblastoma cells. Cell Mol Biol (Noisy-le-grand) 41(4):473–480.

Hallis B, James BA, Shone CC. 1996. Development of novel assays for botulinum type A and B neurotoxins based on their endopeptidase activities. J Clin Microbiol 34(8):1934–1938.

Honer WG, Falkai P, Bayer TA, Xie J, Hu L, Li HY, Arango V, Mann JJ, Dwork AJ, Trimble WS. 2002. Abnormalities of SNARE mechanism proteins in anterior frontal cortex in severe mental illness. Cereb Cortex 12(4):349–356.

Humeau Y, Doussau F, Grant NJ, Poulain B. 2000. How botulinum and tetanus neurotoxins block neurotransmitter release. Biochimie 82(5):427–446.

Jurasinski CV, Lieth E, Dang Do AN, Schengrund CL. 2001. Correlation of cleavage of SNAP-25 with muscle function in a rat model of botulinum neurotoxin type A induced paralysis. Toxicon 39(9):1309–1315.

Koriazova LK, Montal M. 2003. Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. Nat Struct Biol 10(1):13–18.

Le Moine O, Devaster JM, Devière J, Thiry P, Cremer M, Ooms HA. 1994. Trypsin activity. A. New marker of acute alcoholic pancreatitis. Dig Dis Sci 39(12):2634–2638.

Maisey EA, Wadsworth JD, Poulain B, Shone CC, Melling J, Gibbs P, Tauc L, Dolly JO. 1988. Involvement of the constituent chains of botulinum neurotoxins A and B in the blockade of neurotransmitter release. Eur J Biochem 177(3):683–691.

Maksymowych AB, Simpson LL. 2004. Structural features of the botulinum neurotoxin molecule that govern binding and transcytosis across polarized human intestinal epithelial cells. J Pharmacol Exp Ther 310(2):633–641.

Montecucco C, Schiavo G. 1994. Mechanism of action of tetanus and botulinum neurotoxins. Mol Microbiol 13(1):1–8.

Murphy NP, Ball SG, Vaughan PF. 1991. The effect of calcium channel antagonists on the release of [3H]noradrenaline in the human neuroblastoma, SH-SY5Y. Neurosci Lett 129(2):229–232.

Ohishi I, Sakaguchi G. 1977. Activation of botulinum toxins in the absence of nicking. Infect Immun 17(2):402–407.

Pellizzari R, Rossetto O, Schiavo G, Montecucco C. 1999. Tetanus and botulinum neurotoxins: Mechanism of action and therapeutic uses. Philos Trans R Soc Lond B Biol Sci 354(1381):259–268.

Sathyamoorthy V, DasGupta BR. 1985. Separation, purification, partial characterization and comparison of the heavy and light chains of botulinum neurotoxin types A, B, and E. J Biol Chem 260(19):10461–10466.

Schägger H, von Jagow G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166(2):368–379.

Schiavo G, Papini E, Genna G, Montecucco C. 1990. An intact interchain disulfide bond is required for the neurotoxicity of tetanus toxin. Infect Immun 58(12):4136–4141.

Schiavo G, Matteoli M, Montecucco C. 2000. Neurotoxins affecting neuroexocytosis. Physiol Rev 80(2):717–766.

Shone CC, Roberts AK. 1994. Peptide substrate specificity and properties of the zinc-endopeptidase activity of botulinum type B neurotoxin. Eur J Biochem 225(1):263–270.

Simpson LL. 1979. The action of botulinal toxin. Rev Infect Dis 1(4):656–662.

Simpson LL, Maksymowych AB, Park JB, Bora RS. 2004. The role of the interchain disulfide bond in governing the pharmacological actions of botulinum toxin. J Pharmacol Exp Ther 308(3):857–864.

Simpson LL. 2004. Identification of the major steps in botulinum toxin action. Annu Rev Pharmacol Toxicol 44:167–193.

Sloop RR, Cole BA, Escutin RO. 1997. Human response to botulinum toxin injection: Type B compared with type A. Neurology 49(1):189–194.

Stecher B, Gratzl M, Ahnert-Hilger G. 1989. Reductive chain separation of botulinum A toxin—a prerequisite to its inhibitory action on exocytosis in chromaffin cells. FEBS Lett 248(1–2):23–27.

Wang Y, Luo W, Reiser G. 2008. Trypsin and trypsin-like proteases in the brain: Proteolysis and cellular functions. Cell Mol Life Sci 65(2):237–252.