Botulinum neurotoxins (BoNT) are the most potent of all toxins that cause flaccid muscle paralysis leading to death. They are also potential biothreat agents. A systematic investigation of various short peptide inhibitors of the BoNT protease domain with a 17-residue peptide substrate led to arginine-arginine-glycine-cysteine (RRGC) having a basic tetrapeptide structure as the most potent inhibitor. When assayed in the presence of dithiothreitol (DTT), the inhibitory effect was drastically reduced. Replacing the terminal cysteine with one hydrophobic residue eliminated the DTT effect but with two hydrophobic residues made the pentapeptide a poor inhibitor. Replacing the first arginine with cysteine or adding an additional cysteine at the N-terminus did not improve inhibition. When assessed using mouse brain lysates, the tetrapeptides also inhibited BoNT/A cleavage of the endogenous SNAP-25. The peptides penetrated the neuronal cell lines, N2A and BE(2)-M17, without adversely affecting metabolic functions as measured by ATP production, and P-38 phosphorylation. Biological activity of the peptides persisted within cultured chick motor neurons, and rat and mouse cerebellar neurons for more than 40 h, and inhibited BoNT/A protease action inside the neurons in a dose- and time-dependent fashion. Our results define a tetrapeptide as the smallest peptide inhibitor in the backdrop of a large substrate protein of 200+ amino acids having multiple interaction regions with its cognate enzyme. The inhibitors should also be valuable candidates for drug development.

Botulinum neurotoxins (BoNT) produced by strains of Clostridium botulinum, are the deadliest of all toxins (1). There are seven distinct serotypes, (A-F), of which BoNT/A afflicts humans most frequently. The latter is also the most stable in neurons causing long-term paralysis. The toxins are 150 kDa proteins comprising a 50 kDa protease light chain (LC) and a 100 kDa binding-translocating heavy chain (HC). Upon entry to animal body, the toxins travel to peripheral neurons where HC binds and translocates LC into the neurons. After dissociating from HC (2), LC cleaves at specific sites on one of three SNARE proteins, thereby blocking exocytosis of acetylcholine into the neuromuscular junction and causing muscle paralysis. The paralysis prevents normal respiration and eventually leads to death of the animal. One of the hallmark features of BoNT/A intoxication is the extreme persistence of symptoms with neuroparalysis often lasting in excess of 3 to 4 months. Currently, there is no effective postexposure therapeutic available against botulism. Because of extreme toxicity, BoNT is a potential biowarfare and bioterrorism threat, and both Centers for Disease Control and Prevention and the U.S. Department of Agriculture have placed it as a category A threat agent.
Elegant X-ray crystallographic studies by Breidenbach, M. A. and A. T. Brunger (4) revealed the participation of 3 exociste sequences in addition to the cleavable sequence for substrate recognition. On the other hand, optimum length of a peptide inhibitor of BoNT/A was suggested to be of 7 residues long (5). These results suggest further studies be done on the substrate and inhibitor sequences to understand a rather flexible active site structure of the protein (6).

In characterizing a recombinantly produced LC of serotype BoNT/A (LcA), we reported that mercury compounds irreversibly inhibited the endoprotease activity (7). Because mercury is itself toxic, these compounds were not investigated further, but understanding how mercury inhibits LcA activity helped further research into developing other BoNT inhibitors. Several groups reported various small molecules and peptides inhibited BoNT/A protease activity. These include bis-quinolin derivatives (8-10), hydroxamate compounds (11,12), and small cysteinyl peptides (13,14). Most of these compounds, however, function as chelators of zinc, with the potential of nonspecific interaction with many essential cellular Zn-containing proteins. Except for peptides, most of these compounds have limitations due to negligible aqueous solubility that requires the use of organic solvents to dissolve the compound and become problematic in drug development for human use.

While developing a rationale for designing inhibitors of LcA activity, we observed that the arginine at P1’ position must be invariant in the SNAP-25 cleavage site (13). We reasoned that arginine or its derivatives might act as an inhibitor, and indeed discovered that both enantiomers of arginine hydroxamate were inhibitors of LcA activity (15,16). However, the L-arginine hydroxamate with a high $K_i$ of 160 µM was not a great inhibitor. In the current investigation, we described the development of highly effective peptide inhibitors of BoNT/A protease activity. The design of these inhibitors was based on structures of the enzyme and its substrate. These were, the (1) P1’ position of the toxin’s substrate peptide is an essential arginine residue (14,17), and (2) active site and the route leading to the active site are populated by acidic amino acid residues (18,19). From these studies, we reasoned that an arginine derivative and/or a basic peptide should be a good inhibitor of the BoNT/A LC protease domain and investigations supported this reasoning (15,16).

Recently we reported 3-D structures of few of these peptides bound at the active site of LcA (20). In this paper, we describe our systematic studies proving that a tetrapeptide provide an optimum length as the most efficient peptide inhibitor that binds at the active site normally occupied by the substrate. Furthermore, our investigations showed that the peptides survive within neurons for at least 40 h and inhibited BoNT/A activity within two primary neuronal cells without showing any apparent cellular toxicity. These results and the high solubility and solution stability of the peptides, indicated that the peptides would make ideal candidates for BoNT/A drug development. Our demonstration of 4-residue tetrapeptides as highly potent competitive inhibitors highlights the active-site flexibility of these neurotoxins in accommodating much larger substrate sequences.

### EXPERIMENTAL PROCEDURES

**Materials and reagents-** Full-length recombinant BoNT LC protease of serotype A (LcA) and serotype B (LcB) were purified as previously described (21-23). The truncated form of LcA, composed of the first 424 residues (LcA424) was expressed and purified as described (20). A SNAP-25 sequence-derived substrate peptide for LcA (SNKTRIDEANQRATKML) (3,13), a VAMP sequence-derived substrate peptide (24) for LcB (LSELDDRADALQAGAS QFETSAAK LKRKY WWKN LK), both having N-terminal acetylated and C-terminal amidated were custom synthesized and purified to >95% by Quality Controlled Biochemicals (Hampton, MS). Other peptide inhibitors prepared were also amidated at the C-terminus and were custom synthesized and purified to >95% by Quality Controlled Biochemicals (Hampton, MS), and a few by Xenotide LLC, (Vancouver, WA). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA); fetal bovine serum from Hyclone (Logan, UT); and Reagent Pak from Lonza (Walkersville, MD). Polyacrylamide gel electrophoresis (PAGE) and Western blots were
performed using the NuPAGE system (Invitrogen). Supplies included 4X sample buffer, reducing agent, 12% BisTris Gels, MOPS buffer, BisTris transfer buffer, and nitrocellulose blots.

UV-Visible Absorption, and Circular Dichroism Measurements- To determine protein concentration and to assess purity, UV-visible absorption spectra were recorded at 22 °C with a Hewlett-Packard 8452 diode array spectrophotometer. LcA, LcA+Hn, LcA+Hn⁺, LcA+Belt, and BoNT/A concentrations were determined by a colorimetric BCA assay (Pierce) using bovine serum albumin (BSA) as standard. LcA concentration was also determined using A₀.1% (1-cm light path) value of 1.0 at 278 nm (17). Both methods gave the same result. Circular dichroism spectra were recorded at 20 °C (10 °C for Zn-autocatalyzed LC), with a Jasco 718 spectropolarimeter with quartz cuvettes of 1-mm path length. An average of five scans was recorded to increase signal-to-noise ratio at a scan speed of 20 nm/min with a response time of 8 s. For all measurements, a buffer blank was recorded separately and subtracted from sample recordings. Mean residue weights (114.55) were calculated using a molecular mass of 51318.4 Da for the 448-residue LC (17). Secondary structural contents were calculated by SELCON supplied in the Softsec program (Softwood, CO).

Neuronal cell lines- Mouse neuro-2a (N2A) cells and human neuroblast BE(2)-M17 cells were purchased from the American Tissue culture collection (Manassas, VA). N2A cells were cultured in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum (FBS). The (BE-2)-M17 cells were cultured in 1:1 mixture of Dulbecco’s minimum essential medium and F12 medium (DMEM/F12) supplemented with 10% FBS. Cells were removed from flasks following Reagent Pak instructions, resuspended in the appropriate medium to 3.0 X 10⁶ cells/ml, and then added (100 µl/well) to wells of a 96-well microtiter plate. Cells were incubated (37°C, 5% CO₂) 24 h before using the cells to test the effects of tetrapeptides on the cells.

Assessment of Inhibitors by Enzymatic Activity Assays- One unit is defined as the amount of enzyme needed to convert one µmole of substrate into products per ml of reaction mixture per minute. Enzymatic assays were based upon UPLC separation (25) and measurement of the cleaved products originally designed for BoNT/A protease assay using a 17-residue synthetic peptide corresponding to residues187-203 of SNAP-25 (3,7). A master reaction mixture lacking LcA was made and aliquots were stored at -20°C. Stocks (0.005~0.07 mg/ml of LcA prepared in 50 mM Na-HEPES, pH 7.4 containing 0.05% Tween-20) were stored at -20°C. Before each assay, the LcA stock was thawed and diluted in 50 mM HEPES, pH 7.4 containing 0.2 mg/ml BSA. At the time of the assay, 2.5-to 5-µl of LcA stock was added to 25 µl master mix to initiate the enzymatic reaction. Components and final concentration of the 30-µl reaction mixture were 0.9 mM substrate peptide, 0.2 mg/ml BSA, 0.0025 mg/ml LcA, and 50 mM Na-HEPES, pH 7.4. Where indicated, the reaction mixture also contained 0.25 mM ZnCl₂ and 5 mM dithiothreitol.

The amounts of uncleaved substrate and products were measured after separation by reverse-phase UPLC using a Waters Acquity UPLC system equipped with Empower Pro software employing a 1.7-mm C18 column (2.1 X 50 mm) with 0.1% TFA as solvent A and 70% acetonitrile/0.1% trifluoroacetate as solvent B at a flow rate of 0.5 ml/min. The LcA substrate and products were resolved by UPLC with a 0% to 42% B gradient of the solvent over 2 min, followed by column regeneration for 0.7 min. LcB substrate and products were resolved by UPLC with a 0% to 100% B gradient of the solvents over 2 min, held at 100% B for 0.5 min, followed by column regeneration for 0.5 min.

Inhibitor Assessment using Mouse Brain Lysate-Brain (cerebrum and cerebellum) was dissected from adult Balb/c mice. A lysate was prepared by homogenizing mouse brains in a Dounce homogenizer in 10 mM Tris buffered saline (pH 7.4) containing 1 mM sodium fluoride and 5 mM EDTA. After a 30 min incubation (4°C), the lysate was centrifuged (14,000 RPM) for 30 min (4°C). Supernatant fluid was removed and dispersed into 0.5 ml Eppendorf tubes and stored.
Tetrapeptide Inhibitors of Botulinum Neurotoxin

To measure if the inhibitors were able to prevent LcA’s enzymatic activity, inhibitors and LcA were incubated with the brain lysate, and then SNAP-25 cleavage was assessed by Western Blot. Dilutions of inhibitors (0 to 200 µM) and 60 nM LcA were added to mouse brain lysate (78 µg). The samples were incubated for 30 min (37°C) and the reaction stopped by adding sample buffer and reducing agent. After heating (90°C) the samples for 10 min, samples were loaded into wells of a 12% bis tris gel and proteins were separated by electrophoresis (200 V, 1.5 h). Proteins were then transferred onto nitrocellulose membranes and left in Odyssey blocking buffer (LiCOR, Lincoln, NE) overnight (4°C).

SNAP-25 proteins were visualized by incubating the membrane in Odyssey rinse buffer [v:v Odyssey blocking buffer and phosphate-buffered saline containing 0.02% Tween-20 (PBST)] containing SM81, a mouse monoclonal antibody recognizing SNAP-25 (Santa Cruz Biologics, Santa Cruz, CA). The blots were washed in PBST, placed in PBS, and then scanned using the Odyssey Imaging system (LiCOR). Band density (average intensity) was calculated on the Odyssey Infared Imaging software by analyzing the bands concentrations (both uncleaved and cleaved). To determine the percentage of SNAP 25 cleaved by LC’s enzymatic activity, the following formula was used: % cleaved = [(uncleaved band / cleaved band) * 100].

Assessment of Inhibitors using Primary Neuronal cells

Chick Motor neurons- Laminin-coated, 24-well plates were prepared by adding 5 µg/ml of laminin to the wells and incubating (37°C) for at least 1 h. The plates were washed in PBS and dried. The spinal cords from 6-day-old chick embryos were dissected in a single piece. One ml of PBS containing 0.05% trypsin was added to the spinal cords and left for 5 min. The trypsin solution was removed, the spinal cords were gently washed three times in Leibovitz’s L-15 medium. One ml of dissociation solution (1 ml L-15, 100 ml 10% BSA and 1 µl of DNase) then added to each tube. The spinal cords were dissociated by gently triturating the cords eight times using a 1-ml pipette. To the tube was added 0.8 ml of L15-C medium to the remaining 0.5-ml spinal cord cell suspension and triturated the mixture eight more times or until the solution appeared homogeneous with no apparent tissue chunks. The cells were resuspended in 1–1.5 ml of the 4% BSA solution, centrifuged for 10 min at 230 x g. The medium was removed and the cell pellet resuspended to 150,000 cells per well in 500 µl of medium. The cells were grown for 3 days then treated with BoNT/A holotoxin with or without prior treatment with inhibitors.

Primary rat and mouse cerebellar neurons- Neuronal cerebellar granule cells were isolated from 7-8-day-old rat or mice (CD-1) as previously described (26). Cells were suspended in DMEM-F-12 medium (Gibco-Invitrogen, Carlsbad, CA) supplemented with 50 U/ml penicillin, 50 µg/ml of streptomycin, 10% FBS, N2 supplement, and 25 mM KCl. Cells were seeded onto poly(L-lysine)-coated 24-well plates at a density of 1 x 10^5 cells per well and were maintained in a humidified 5% CO₂ atmosphere at 3°C. After 24 h, 10 µM cytosine-B-D-arabinoside (Sigma, St. Louis, MO) was added to the culture to inhibit replication of non-neuronal cells. DMEM/F12 B27 medium containing B27 supplement (Invitrogen) was replaced every 7 days. The primary neuronal cell cultures were well-established 8-10 days after initial seeding and remained viable for 2 weeks.

To test the inhibitory effects of the peptides on BoNT/A activity within the neuron, varying concentrations of inhibitors were added to the cultures and incubated overnight (16 h). The cultures were then repeatedly washed with the medium to remove the inhibitors from the medium or the cell surface. After the addition of 4 nM (chick neurons), 1 nM (rat neurons), or 0.1 nM (mouse neurons) BoNT/A or 1 nM BoNT/B (mouse neurons), the cells were incubated for 2, 4 or 24 h (37°C, 5 % CO₂). Volume of the medium per well in the 24 h BoNT/A or BoNT/B incubation was twice that for 2h and 4 h incubations. Before and after each stage of treatment with inhibitors and BoNT/A, the plates were examined under a light microscope for cell morphology. The cells were washed with PBS, harvested into preweighed Eppendorf screw-cap vials, and then pelleted by centrifugation (230 x g,
The supernatant fluid was carefully removed and the microcentrifuge tubes were weighted to determine the weight of the pellet. Four µl of sample, and 2 µl of 3x SDS-PAGE sample buffer were added for each mg of cell pellet. Samples were heated (90°C) for 10 min to inactivate any residual toxin, and then analyzed for SNAP-25 cleavage by Western blot using rabbit anti-SNAP-25 IgG (Sigma, St. Louis, MO) or anti-VAMP-2 rabbit polyclonal (Thermo Fisher Scientific PA1-28308). Western blot band densities for SNAP-25 and VAMP-2 products were normalized and relative intensity was determined using scanning densitometry (Kodak Image station 200RT, Eastman Kodak Company, Rochester, NY).

**SNAP25 Western blot** analysis was performed using 12% polyacrylamide gels and then proteins were transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA). Unbound sites were blocked by incubating the membranes in TBST (10 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween 20) containing 5% nonfat dry milk (TBST-5% milk) for 1 h (25°C). Membranes were probed with rabbit anti-SNAP-25 IgG (Sigma, St. Louis, MO) in TBST-1% milk overnight (25°C). Membranes were washed in TBST and then incubated (25°C) in TBST-5% milk containing goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences). Membranes were washed in TBST and SNAP-25 detected using an ECL kit (GE Healthcare Bio-Sciences Corp.). Band densities for SNAP-25 product were normalized and relative intensity was determined using scanning densitometry (Kodak Image station 200RT, Eastman Kodak Company, Rochester, NY).

**Assessment of Toxicity of Inhibitors in Cell Culture** - For cell viability assays, N2A and BE(2)-M17 cells were cultured in 96-well cell culture, flat-bottom plates (37°C, 5% CO2). The inhibitors were diluted in serum-free medium that was then added (100 µl/well) to the wells, and the plates returned to the incubator. A negative cytotoxic (cells only) and a positive cytotoxic control (6 µM SDS) were included on the plate. After 24 h, ATP release was measured using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison WI) following the vendor’s instructions. Briefly, 100 µl of cell Glo reagent was added to each well and 15 min later, luminescence was measured on a Victor Wallac multiplate reader (Perkin Elmer, Waltham, MA).

Phosphorylation of p38 MAPK within the neuronal cells was monitored using the RayBio® Cell-Based p38 MAPK (Thr180/182) ELISA kit (Ray Biotech, Inc., Norcross, GA) following vendor’s instructions. Briefly, inhibitors (600, 60, and 6 µM final concentrations) were diluted in medium, added to cells in a 96 well plate and placed in an incubator (37°C, 5% CO2). Phorbol 12-myristate 13-acetate (PMA) was included for a positive control to show activation p38 activation (27). After 30 or 60 min, plates were washed gently followed by fixing the cells to the plate. Detection of p38 and phosphorylated p38 was monitored by probing the cells with manufacturer-supplied anti-p38 and anti-phosphorylated p38 primary antibodies, counterstaining with a HRP conjugated to anti-IgG secondary antibody. Color was developed by adding TMB one-Step substrate reagent, and absorbance read at 450 nm in the Victor plate reader. In addition to viability assays and the MAPK assays, cell morphology was observed using an inverted light microscope and showed that addition of inhibitors did not affect cell morphology or monolayer development (data not shown).

**RESULTS**

**Tetrapeptides as Inhibitors of BoNT/A Proteolytic Activity** - Previously, we reported on designing basic compounds and basic peptides as BoNT/A LC protease inhibitors (15,16). Our studies showed that protamine sulfate and a HIV-tat-derived peptide, GRKKRRQRRRPPQC, inhibit 80-90% of BoNT/A LC activity while an eight-residue polyarginine peptide is less effective (15,16). Table I and Figure 1A show that the human immunodeficiency virus (HIV)-tat peptide has a very low $K_i$ of 96 nM, one of the lowest described in literature (8-12,14,28,29). The HIV-tat peptide and the polyarginine peptide, however, are known to readily penetrate plasma membranes of various mammalian cells (30,31) with a potential to affect cellular functions and making them less likely candidates for drug development. In order to design a peptide that would have a better BoNT/A inhibition with reduced cell penetration, we first chose to vary the length of the
arginine peptide by increasing or decreasing the number of arginine residues. Table I shows that a tetrapeptide having two N-terminal arginines provided the greatest inhibition of BoNT/A. Interestingly, removal of one arginine from this peptide resulted in complete loss of the inhibitory property.

To improve the inhibitor further, we varied the sequence of the tetrapeptide. Table II shows that the C-terminal cysteine in RRGC could be replaced by one (RRGL, RRGI, RRGM and RRGF) but not by two hydrophobic (RRGLL and RRGAL) residues nor by a polar (RRGT) residue. Similarly, from a comparison of inhibitions by RRAT and RRGT, glycine as the third residue in the tetrapeptide appears to make it the most effective inhibitor. Our recent X-ray crystallographic studies showed that the inhibitor RRGC mimicked the substrate sequence QRAT in having the Q-R cleavable bond, and the essentially identical interactions at the active site of BoNT/A LC (20,32).

There are several hydrophobic residues (I161, F163, F194) in the pocket occupied by the second arginine of the inhibitor or substrate (20,32) offering a possibility that a hydrophobic residue in this position might also make effective inhibitors. Similarly, the structure of substrate QRATKM-LcA complex showed a deep polar pocket around the third residue, alanine (A) (32) that suggests the possibility of accommodating a bulkier residue at this position. Moreover, from comparison of several inhibitor-LcA structures (6), Silvaggi et al noted that there are sufficient opportunities of alternate interactions at the active site (33). However, changing the second arginine to a hydrophobic tryptophan residue (in RWGC) or by changing the second and third residues to hydrophobic residues of tyrosine-phenylalanine (in RYFC) did not improve the inhibition (Table II). Failure to improve inhibition may be related to the fact that the side chain of the second residue has the freedom of taking one of two alternate positions as has been observed with RRGI and RRGC (or RRGL and RRGM)-LcA structures (20). However, this result is consistent with the absolute requirement of arginine at the p1’ position of the substrate (13). Our LcA-RRGC structure also showed that beside a salt bridge between the first arginine of the inhibitor and E164 of the protein, there is a space occupied by six to eight water molecules (20) that may be replaced by a bulkier amino acid residue. There is a possibility that replacing the first arginine with tryptophan or tyrosine although will eliminate the salt bridge, it may give a better spatial fit and a stacking interaction with a nearby histidine 227 (20). Moreover, Schmidt and Stafford (34) showed that a cysteine at the N-terminus of a heptapeptide (CRATKML) was essential for inhibition of LcA activity. We therefore replaced this arginine with tryptophan (WRGC) and cysteine (CRGC), exchanged the positions of terminal arginine and cysteine (CRGF from RRGF), and added an extra cysteine at the N-terminus (of CRRGC and CRRGF). None of these changes improved the inhibitor activity (Table II). These results show that although changes in the p1 residue is tolerated in the substrate sequence (3,13), it makes the tetrapeptide a less effective inhibitor.

The above results firmly established the basic tetrapeptides RRGC, RRGL, RRGI, RRGM, RRGF, and CRGF as the shortest peptides mimicking the substrate that can act as the most effective inhibitor of BoNT/A LC. All of these peptides inhibited LcA activity competitively with substrate (Figure 1A-F). The Ki, 157~850 nM for these peptides (Figure 1B and Table II) place them as a class of inhibitors among the highest-affinity BoNT inhibitors (8-12,28,29,34). Only one inhibitor was very recently reported (29) with a Ki lower than 157 nM (Table II). Although the HIV-tat derived peptide displayed a smaller Ki, because of its potential toxicity, we did not investigate it further. As shown in Table III, IC50 of RRGC or any of its enantiomeric variants was dramatically increased by adding DTT to the assay mixtures and suggested that RRGC might not be an ideal drug candidate as the reducing cellular environment could conceivably diminish its effectiveness. Because the tetrapeptides RRGL, RRGI, and RRGM were not affected by the presence of DTT (Table III), the presence of a hydrophobic residue at the C-terminus would be ideal as potential drug candidates by stabilizing the inhibitor within the cellular microenvironment.

Specificity of Tetrapeptides— Each of the seven BoNT serotypes have strict substrate specificity (1). Because our tetrapeptides resemble the BoNT/A substrate both in sequence and in...
molecular interactions (20,32), the peptides should be expected to be specific inhibitors of BoNT/A and not affect other BoNT serotypes. While the active-site geometry of the BoNT serotypes is similar (32,35,36), each cleaves a specific site in the substrate. For example, the cleavable bond of the BoNT/A substrate is Q-R while that of BoNT/B substrate is Q-F. As a test case for specificity, we compared the ability of RRGC in inhibiting BoNT/B LC activity. At 20 \( \mu \text{M} \) concentration, RRGC inhibited 95% of BoNT/A LC activity while inhibiting only 7% of BoNT/B LC activity. Increasing the inhibitor concentration tenfold resulted in 100% inhibition of LcA activity, but only a 20% decrease in LcB activity. Because the active site for BoNT/B is similar to BoNT/A, the lack of significant LcB inhibition by RRGC, suggested that the RRGC is LcA specific. Specificity of the tetrapeptides for BoNT/A were confirmed with mouse cerebellar granules (see later). While the peptides specificity for LcA might be problematic in development of a generalized inhibitor for BoNTs in general, the specificity of the tetrapeptide indicated that it would not inhibit other intracellular proteases required for cellular processes and therefore, would not have adverse effects on the neurons.

Inhibitor Affinity Determination efforts- Although the \( K_i \) values reported in Table II provide an indication of inhibitor effectiveness, they are not a true measure of affinity. Recently, Burnett and coworkers (5) reported determination of affinity constant \( K_a \) for BoNT/A LcA by a pseudopeptide using isothermal titration microcalorimetry. This technique was not used for confirmation the studies described here because of the inherent instability of BoNT domains towards mild agitation (37) that is necessary in the microcalorimetry technique. In addition, the metallic needles in such instruments are likely to catalyze an autocatalytic fragmentation reaction of BoNT/A LcA (21,38) that would almost certainly affect the \( K_a \) determination. Therefore other methods were explored to detect biophysical parameter changes in BoNT/A LcA that might accompany the inhibitor binding. We measured the far-UV circular dichroism of the protein in the presence and absence of 20 \( \mu \text{M} \) inhibitors in 50 mM Na-phosphate, pH 6.8, as a function of temperature. While there was no significant difference in the CD spectra (data not shown), RRGC, RRGL, RRGI, RRGM, and RRGF increased \( T_{m} \), the midpoint of thermal denaturation (measured at 220 nm) by 0.2–3 °C. Thus binding of inhibitors increased the midpoint of thermal denaturation of BoNT/A LC. However, due to low instrument sensitivity (in accurately controlling temperature less than 0.2°C), such a small change in this parameter was not explored further for inhibitor affinity, \( K_a \) determinations.

Inhibition of SNAP-25 cleavage by BoNT/A- The above inhibition studies used a 17-residue peptide of the much larger SNAP-25 natural substrate. Further studies were conducted in which inhibition of LcA activity was examined using the native SNAP-25 substrate in mouse brain lysates. In this assay too, the tetrapeptides inhibited the BoNT/LcA-catalyzed cleavage of SNAP-25 (Fig. 2). For comparison, in this experiment we also included the classic heptapeptide inhibitor of BoNT/A (14). In addition, RRGL was shown to inhibit at a lower concentration than the RRGC peptide. This result was consistent with those presented in Table III. Such results were also expected because the total rat brain extract contains many reducing agents such as glutathione and cysteine that would form mixed disulfides with RRGC but not with the RRGL. As a result, the effective concentration of RRGC would be much lower than that of RRGL. While RRGL was a better inhibitor than RRGC, both were effective in situ and were not adversely affected by numerous cellular components. In addition to these two peptides, two other peptides, RRGI and RRGM, were also found to inhibit LcA activity in mouse brain lysates.

Inhibition of BoNT/A activity within chick, rat and mouse neuronal cells- To test the ability of the tetrapeptides to penetrate the neuronal cells, we incubated chick motor neurons with various concentrations (20 \( \mu \text{M} \) to 600 \( \mu \text{M} \)) of RRGC for 24 h. After removing the excess inhibitor by repeated washing, the cells were incubated with 4 nM BoNT/A. The cells were lysed after 2 h, 4 h and 24 h, and the full-length SNAP-25 and its cleaved products were visualized by western blot using anti-SNAP-25 IgG. As a loading control for the 24 h sample, VAMP-2 was also detected. As shown in Figure 3, increasing concentrations RRGC inhibited BoNT/A activity within the chick motor neurons such that at 600 \( \mu \text{M} \), 91%, 86%, 65% BoNT/A activity was inhibited compared to...
BoNT/A alone at 2 h, 4 h, and 24 h respectively. Moreover, at the lowest concentration of 20 μM, ~80-60% of inhibition was observed during 2-4 h BoNT/A treatment. These results indicated that the inhibitor was able to penetrate the chick neuronal cells and prevent LcA activity within the neuronal cell. Furthermore, the inhibitor appeared to remain within the cell for at least 24 h as approximately the same levels of inhibition could be detected in cells treated with BoNT/A for 24 h at higher inhibitor concentrations (lower panel, Figure 3). This result also suggested that BoNT/A-RRGC complex was stable within the cells for at least 24 h. Additionally, these results suggested that the RRGC inhibitor was stable to intracellular protease action for 24-40 h.

In addition to assessing the inhibitors effectiveness within chick motor neurons, primary rat and mouse cerebellar neuronal cells were also used to assess the inhibitory properties. As shown in Figure 4A, RRGL, RRGM, RRGF, CRGF, and CRGC provided more inhibition than did RRGC. Much higher inhibition by RRGL than RRGC of BoNT/A LC activity in the presence of DTT (Table III), and of BoNT/A activity rat brain extract (Figure 2) described above were also supported by higher inhibition in this rat cerebellar neurons. The most notable feature of this experiment was that CRGC had the highest inhibitory property in neurons, although it was not the best inhibitor in in vitro assay using the synthetic 17-residue peptide substrate (Table II). We do not have an explanation for the enhanced inhibition by CRGC in the cellular environment, but structural data may suggest additional interaction of the inhibitor with the protein residues.

To test the specificity of the peptides for inhibition of BoNT/A, mouse granule neurons were prepared and treated for 4 hours with the best in vitro and in vivo inhibitors (RRGC and CRGC) against BoNT/A and BoNT/B activity. In Figure 4B, cleavage of SNAP-25 by BoNT/A is indicated by appearance of a second band of lower molecular weight on western blot. In the case of BoNT/B cleavage of VAMP-2, the immunoreactive band for VAMP-2 disappeared with cleavage. After only 4 hour treatment with 600 μM, CRGC completely and RRGC almost completely inhibited SNAP-25 cleavage by BoNT/A, but VAMP-2 levels remained unchanged by the peptide treatments. When the cells were treated with BoNT/B (Figure 4B, lower panel), neither RRGC nor CRGC prevented VAMP2 cleavage by BoNT/B. These results clearly demonstrate that RRGC and CRGC are specific inhibitors of BoNT/A (and not of BoNT/B) in cellular environments, as was observed before with RRGC in the 17-mer peptide substrate in vitro assay (see above).

Evaluation of Cytotoxicity of the Tetrapeptides-Tolerability of the inhibitors within cells was examined using mouse neuroblastoma N2A cells, human neuroblastoma BE(2)-M17 cells, and rat cerebellar primary cells were examined under light microscopy before and after treatment with the inhibitors. No morphological change was observed after the treatment and the cells had typical central cell body with extended dendrites. There was no rounding of the cells, detachment, or retraction of cellular processes.

In order to determine whether the peptides were cytotoxic, we tested N2A and BE(2)-M17 cells using a commercially available kit that measures ATP by a luminescent reaction. Measuring ATP activity provides a sensitive assay that is used as an indicator of metabolically active and viable cells (39-41). Viable cells contain active ATP while dead cells do not, so that a decrease in luminescence measured as counts per second (CPS), indicated less active ATP and a loss of cell viability. (39-41). Results (Figure 5) were expressed as CPS treated cells/CPS control cells X 100 to give the percent of the cell control. SDS was included in the experiment as a positive cytotoxic cell control. Results of treating both cell lines with the peptide inhibitors showed little difference between the treated cells and the untreated controls indicating the inhibitors did not kill the cells and suggested no adverse effect in cell viability by any of the inhibitors (Figure 5). In contrast, including the cytotoxic SDS drastically reduced light production. Along with the findings described in Figures 3-5, these results suggested there was rapid uptake of the inhibitors by the neuronal cells and the inhibitors were not toxic to the cells.

Another biochemical parameter, activation of MAP kinase transduction pathways is widely used to monitor cellular responses to exogenous agents that might affect the cell intracellularly (42-45). N2A and BE (2)-M17 cells were treated with
the inhibitors RRGC, RRGL, RRGI and CRGC, and incubated (37°C, 5% CO2) for 30 and 60 min at which time the cells were fixed and then assessed using an in cell Western assay. Normally, p38 becomes phosphorylated as a consequence of cellular activation and proliferation. Phosphorylation of p38 was not increased when treated with 600 µM (Figure 6A) or at lower concentrations, 60 µM (not shown) and 6 µM (Figure 6B). A comparison of the results at the high and low inhibitor concentrations does not indicate significant differences. In contrast, phosphorylated MAPK p38 increased after 60 min incubation with phorbol myristate acetate (26) indicating that the assay detected activation of p38 (Figure 6, + control). Although RRGC showed slightly higher levels than control, phosphorylated p38 was not higher than the nonphosphorylated counterpart. Absence of increase in phosphorylated p38 by treatment of BE (2)-M17 (Figure 6) and N2A (not shown) with all three concentrations of the inhibitors suggested that the tetrapeptides did not affect the cells signaling pathways and therefore would not damage the cells.

Solubility and Stability - Unlike most small molecule compounds (8-12), the tetrapeptides are highly soluble in water. We routinely made 40 mM solutions of the tetrapeptides in Bis-Tris buffer pH 6.8 (20) without any sign of precipitation. Similarly, 0.02~2.0 mM solutions of the tetrapeptide in distilled water or in 50 mM HEPES buffer, pH 7.4 was stored at -20°C for over a year without any apparent decrease in their inhibition of LcA protease activity (data not shown). This result suggested that the peptides were stable in solution over this extended period.

DISCUSSION

Botulinum neurotoxins have long been known to be unique among proteases in requiring a large substrate sequence (1,46). This uniqueness is also supported by recent X-ray crystallographic studies (4) that pinpointed at least 3 exosite sequences in addition to the sequence containing the cleavable bond in the BoNT/A substrate SNAP25. In addition, from substrate sequence based inhibitor development efforts, a minimum of 7 residue was predicted as optimal for an active site inhibitor (5,14). By systematic variation of the length of a basic peptide, in this study we demonstrated that a tetrapeptide is much better an inhibitor of BoNT/A active site than any other peptide described by others thus far. Our results prove that although a large amino acid sequence is required by BoNT/A, a much smaller tetrapeptide can function as a potent competitive inhibitor. Our results support the idea of active site structural flexibility of BoNT/A (33) and probably of all other serotypes of BoNT. By modifying our basic tetrapeptide structures, it may be possible to further develop much higher affinity inhibitors.

Ever since the danger of BoNT as a potential biothreat agent was recognized, a post exposure therapeutic development effort was initiated. Since the anthrax attacks of 2001-02, a renewed effort of therapeutic development has mainly focused on small molecules as BoNT protease inhibitors. High-throughput screening of ~70,000 compounds in the NCI diversity set (10) and combinatorial chemistry-generated libraries (47,48), and virtual screens of NCI database (49) has identified few small molecules as LcA protease inhibitors with $K_i$ mostly in the 1-20 µM range compared to our sub-micromolar results. Although 2,4-dichlorocinnamic hydroxamate was initially reported to have $K_i$ value of 0.3 µM (50), more recently $IC_{50}$ values of 15-81 µM were reported depending on assay conditions (51,52).

A molecular dynamics simulations in a cationic dummy atom approach developed an inhibitor with $K_i$ of 4 µM (53) that was later improved to 0.8 µM by synthesis-based computer-aided molecular design (54), and a recent pharmacophore-guided lead inhibitor optimization achieved an inhibitor $K_i$ of 0.6 µM (55). All of these small molecules (53-55) (mass ~600 daltons) contain one or more bicyclic aromatic rings making them extremely hydrophobic and thus requiring solubility in organic solvents that potentially could have adverse effect on cells. The chemical nature of some compounds that were shown to have effectiveness in neuronal N2A cells are not reported (48). Although inhibition by most of these compounds was stated to be competitive with the substrate, three-dimensional structural information is currently not available and therefore, the exact location of their binding to the target protein is not known.
In this investigation, we showed that small basic peptides such as a tetrapeptide (mass ~500 daltons) were highly potent inhibitors of BoNT/A protease activity and possess $K_i$ smaller than (160, 360 nM) or equal to (664~800 nM) those reported with organic small molecule inhibitors (53-55).

In addition to solubility, the small hydrophobic molecules will invariably require a carrier molecule for the inhibitor to gain entry into the cell. With a cationic character and being highly soluble in aqueous solvents, the cationic peptides were shown to penetrate neuronal cell lines N2A and BE(2)-M17 as well as primary chick motor neurons, and rat and mouse cerebellar neurons, thus alleviating the need for a helper or carrier molecule. The pioneering work of J. J. Schmidt established the use of substrate-based peptides as BoNT inhibitors (17). The CRATKML heptapeptide is currently used as a reference inhibitor even though the peptide has a relatively high $K_i$ of 1.8 µM (17). Later optimization in which the first residue was replaced with a nonprotein adduct (2-mercapto-3-phenylpropionyl group), improved the $K_i$ to 330 nM (14). More recently a dinitrophenyl-diaminobenzoate peptidomimetic of the original CRATKML improved the $K_i$ to an unprecedented 41 nM (29). However, if these peptides can penetrate neuronal cell membranes and if they are active and nontoxic inside the cells is not known. Moreover, the only other known three-dimensional (besides those of the tetrapeptide inhibitors (20,56)) LcA- inhibitor (peptidomimetic) structure (29), clearly show that the inhibitor occupied positions at the active site other than those normally occupied by the substrate peptide. Our arginine-containing tetrapeptide inhibitors (20,56) and the first four residue of the true substrate QRATKM (32) occupies essentially identical positions at the active site, confirming our solution studies that they are the true competitive inhibitors. Thus, on a structural perspective, the peptidomimetic (29) is not a true competitive inhibitor when compared to the tetrapeptide inhibitors.

A major concern for the use of peptides as drug candidates is their short intracellular half life (57). However high activity, high specificity, high potency, low toxicity, low drug-drug interaction, low tissue accumulation, recent improvements in formulation, and manufacturing technology are increasingly bringing peptides into the drug market (58,59). We have shown that our peptides survived 24–40 h in chick motor, and in rat and mouse cerebellar neurons. Thus, these peptides appear to be resistant to intracellular protease action while not adversely affecting the cell’s metabolism, making them potential candidates for drug development. By keeping the second amino acid constant as arginine, first residue as cysteine or arginine and the fourth residue as hydrophobic in these tetrapeptides, we have ensured that they mimic the natural substrate sequence and act as truly competitive inhibitors. Finally, structural proof of our peptides as competitive inhibitors, and demonstration that a four-residue peptide was the optimum length, was supported by three-dimensional structure determination of LcA in complex with eight tetrapeptides (20,56) and two hexapeptides (32).

**In summary**, four-residue tetrapeptides with an arginine at the second position represent an optimum length and sequence, and acts as potent competitive inhibitors of BoNT serotype A. These peptides have solubility, specificity, intracellular stability, nontoxicity and BoNT/A inhibitory properties consistent with drug development characteristics.

**REFERENCES**

1. Simpson, L. L. (2004) *Annu Rev Pharmacol Toxicol* **44**, 167-193
2. Gul, N., Smith, L. A., and Ahmed, S. A. (2010) *PLoS ONE* **5**(9), e12872
3. Schmidt, J. J., and Bostian, K. A. (1995) *J Protein Chem* **14**(8), 703-708
4. Breidenbach, M. A., and Brunger, A. T. (2004) *Nature* **432**(7019), 925-929
5. Burnett, J. C., Ruthel, G., Stegmann, C. M., Panchal, R. G., Nguyen, T. L., Hermone, A. R., Stafford, R. G., Lane, D. J., Kenny, T. A., McGrath, C. F., Wipf,
Tetrapeptide Inhibitors of Botulinum Neurotoxin

P., Stahl, A. M., Schmidt, J. J., Gussio, R., Brunger, A. T., and Bavari, S. (2007) *J Biol Chem* **282**(7), 5004-5014

6. Silvaggi, N. R., Boldt, G. E., Hixon, M. S., Kennedy, J. P., Tzipori, S., Janda, K. D., and Allen, K. N. (2007) *Chem Biol* **14**(5), 533-542

7. Ahmed, S. A., and Smith, L. A. (2000) *J Protein Chem* **19**(6), 475-487

8. Sheridan, R. E., Deshpande, S. S., Nicholson, J. D., and Adler, M. (1997) *Toxicon* **35**(9), 1439-1451

9. Burnett, J. C., Opsenica, D., Sriraghavan, K., Panchal, R. G., Ruthel, G., Hermone, A. R., Nguyen, T. L., Kenny, T. A., Lane, D. J., McGrath, C. F., Schmidt, J. J., Vennerstrom, J. L., Gussio, R., Solaja, B. A., and Bavari, S. (2007) *J Med Chem* **50**(9), 2127-2136

10. Burnett, J. C., Schmidt, J. J., Stafford, R. G., Panchal, R. G., Nguyen, T. L., Hermone, A. R., Vennerstrom, J. L., McGrath, C. F., Lane, D. J., Sausville, E. A., Zaharevitz, D. W., Gussio, R., and Bavari, S. (2003) *Biochem Biophys Res Commun* **310**, 84-93

11. Capkova, K., Yoneda, Y., Dickerson, T. J., and Janda, K. D. (2007) *Bioorganic & medicinal chemistry letters* **17**(23), 6463-6466

12. Boldt, G. E., Eubanks, L. M., and Janda, K. D. (2006) *Chemical communications (Cambridge, England)* (29), 3063-3065

13. Schmidt, J. J., and Bostian, K. A. (1997) *J Protein Chem* **16**(1), 19-26

14. Schmidt, J. J., and Stafford, R. G. (2002) *FEBS Lett* **532**(3), 423-426

15. Ludivico, M., Smith, L. A., and Ahmed, S. A. (2006) Amino Acids and Peptides as Inhibitors of Botulinum Neurotoxin Serotype A Proteolytic Activity. In: Keller, J. E. (ed). *43rd Annual Meeting of the IBRCC*, CBER, FDA, Silver Spring, MD

16. Ludivico, M., Smith, L. A., and Ahmed, S. A. (2009) *The Botulinum Journal* **1**(3), 297-308

17. Schmidt, J. J., Stafford, R. G., and Bostian, K. A. (1998) *FEBS Lett* **435**(1), 61-64

18. Lacy, D. B., and Stevens, R. C. (1999) *J Mol Biol* **291**(5), 1091-1104

19. Lacy, D. B., Tepp, W., Cohen, A. C., DasGupta, B. R., and Stevens, R. C. (1998) *Nat Struct Biol* **5**(10), 898-902

20. Kumaran, D., Rawat, R., Ludivico, M. L., Ahmed, S. A., and Swaminathan, S. (2008) *J Biol Chem* **283**(27), 18883-18891

21. Ahmed, S. A., McPhie, P., and Smith, L. A. (2003) *Biochemistry* **42**(43), 12539-12549

22. Jensen, M. J., Smith, T. J., Ahmed, S. A., and Smith, L. A. (2003) *Toxicon* **41**(6), 691-701.

23. Gilsdorf, J., Gul, N., and Smith, L. A. (2006) *Protein Expr Purif* **46**(2), 256-267

24. Foran, P., Shone, C. C., and Dolly, J. O. (1994) *Biochemistry* **33**(51), 15365-15374

25. Rowe, B., Schmidt, J. J., Smith, L. A., and Ahmed, S. A. (2010) *Anal Biochem* **396**(2), 188-193

26. Kuo, C. L., Oyler, G., and Shoemaker, C. B. *Toxicon* **55**(2-3), 619-629

27. Awasthi, V., and King, R. J. (2000) *Am J Physiol Lung Cell Mol Physiol* **279**(5), L942-949

28. Schmidt, J. J., and Stafford, R. G. (2005) *Biochemistry* **44**(10), 4067-4073
29. Zuniga, J. E., Schmidt, J. J., Fenn, T., Burnett, J. C., Arac, D., Gussio, R., Stafford, R. G., Badie, S. S., Bavari, S., and Brunger, A. T. (2008) *Structure* 16, 1588–1597
30. Suzuki, T., Futaki, S., Niwa, M., Tanaka, S., Ueda, K., and Sugiura, Y. (2002) *The Journal of biological chemistry* 277(4), 2437-2443
31. Nakase, I., Takeuchi, T., Tanaka, G., and Futaki, S. (2008) *Advanced drug delivery reviews* 60(4-5), 598-607
32. Kumaran, D., Rawat, R., Ahmed, S. A., and Swaminathan, S. (2008) *PLoS Pathog* 4(9), e1000165
33. Silvaggi, N. R., Wilson, D., Tzipori, S., and Allen, K. N. (2008) *Biochemistry* 47(21), 5736-5745
34. Schmidt, J. J., and Stafford, R. G. (2002) *FEBS Lett* 532, 423-426
35. Agarwal, R., and Swaminathan, S. (2008) *J Biol Chem* 283(38), 25944-25951
36. Sikorra, S., Henke, T., Swaminathan, S., Galli, T., and Binz, T. (2006) *J Mol Biol* 357(2), 574-582
37. Toth, S. I., Smith, L. A., and Ahmed, S. A. (2009) *Journal of pharmaceutical sciences* Published online Feb 18, 2009
38. Ahmed, S. A., Ludivico, M. L., and Smith, L. A. (2004) *Protein J* 23(7), 445-451
39. Saikh, K. U., Kissner, T., and Ulrich, R. G. (2002) *Immunology* 106(3), 363-372
40. Doherty, D. G., Norris, S., Madrigal-Estebas, L., McEntee, G., Traynor, O., Hegarty, J. E., and O'Farrelly, C. (1999) *J Immunol* 163(4), 2314-2321
41. Vylkova, S., Sun, J. N., and Edgerton, M. (2007) *Purinergic Signal* 3(1-2), 91-97
42. Morinobu, A., Gadina, M., Strober, W., Visconti, R., Fornace, A., Montagna, C., Feldman, G. M., Nishikomori, R., and O'Shea, J. J. (2002) *Proc Natl Acad Sci U S A* 99(19), 12281-12286
43. Zarubin, T., and Han, J. (2005) *Cell Res* 15(1), 11-18
44. Lee, S. Y., Lee, J. W., Lee, H., Yoo, H. S., Yun, Y. P., Oh, K. W., Ha, T. Y., and Hong, J. T. (2005) *Brain Res Mol Brain Res* 140(1-2), 45-54
45. Goldstein, D. M., and Gabriel, T. (2005) *Curr Top Med Chem* 5(10), 1017-1029
46. Tonello, F., Morante, S., Rossetto, O., Schiavo, G., and Montecucco, C. (1996) *Adv Exp Med Biol* 389, 251-260
47. Boldt, G. E., Kennedy, J. P., Hixon, M. S., McAllister, L. A., Barbieri, J. T., Tzipori, S., and Janda, K. D. (2006) *Journal of combinatorial chemistry* 8(4), 513-521
48. Eubanks, L. M., Hixon, M. S., Jin, W., Hong, S., Clancy, C. M., Tepp, W. H., Baldwin, M. R., Malizio, C. J., Goodnough, M. C., Barbieri, J. T., Johnson, E. A., Boger, D. L., Dickerson, T. J., and Janda, K. D. (2007) *Proc Natl Acad Sci U S A* 104(8), 2602-2607
49. Roxas-Duncan, V., Enyedy, I., Montgomery, V. A., Eccard, V. S., Carrington, M. A., Lai, H., Gul, N., Yang, D. C., and Smith, L. A. (2009) *Antimicrobial agents and chemotherapy* 53(8), 3478-3486
50. Boldt, G. E., Kennedy, J. P., and Janda, K. D. (2006) *Organic letters* 8(8), 1729-1732
51. Capkova, K., Salzameda, N. T., and Janda, K. D. (2009) *Toxicon* 54(5), 575-582
52. Pires-Alves, M., Ho, M., Aberle, K. K., Janda, K. D., and Wilson, B. A. (2009) *Toxicon* 53(4), 392-399
ACKNOWLEDGMENTS

We thank Mr. Matthew Ludivico for conducting the UPLC™ analyses, kinetic and circular dichroism experiments, Ms. Azkia Mujib for rat brain extract assays, and Ms. Michelle Saylor for cytotoxicity assays. We also thank Drs. Kamal Saikh for guidance with the cytotoxicity assays, and Michael Lee for help in graphic displays. This project received support from the Defense Threat Reduction Agency-Joint Science and Technology Office for Chemical and Biological Defense (Grant #s CBS.MEDBIO.01.10.RD.002 and JSTOCBD3.10012_06_RD_B to SAA). Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Abbreviations used: BoNT, botulinum neurotoxin; BoNT/A, botulinum neurotoxin serotype A; BoNT/B, botulinum neurotoxin serotype B; LC, light chain; LcA, light chain of serotype A; LcB, light chain of serotype B; HC, heavy chain; Hn, N-terminal domain of the heavy chain; Hc, C-terminal domain of the heavy chain; SNAP-25, synaptosomal-associated protein of 25 kDa; VAMP, vesicle associated membrane protein; SNARE, soluble NSF attachment protein receptor; EDTA, ethylenediamine tetraacetate; SDS, sodium dodecyl sulfate, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; MES, 2-[N-morpholino]ethanesulfonic acid.
TABLE I: Optimum length of the arginine peptide as an inhibitor

| Peptide                  | %Activity |
|--------------------------|-----------|
| None                     | 100       |
| GRKKRRQRRPPQC            | 10 (Ki = 96±57 nM\(^1\)) |
| RRRRRRRRGGC              | 30        |
| RRRRRRRRRRRRRRRGCG       | 40        |
| RRRRRRRRRRRRRRRRGGC      | 30        |
| RRRRRRRRRRRRRRRRGGC      | 20        |
| RRRRRRRRRRRRRRRRGGC      | --        |
| RRRRRRRRRRRRRRGGC        | 20        |
| RRRRRRRRRRRRGGC          | 15        |
| RRRRRRRRRRRGGC           | 20        |
| RRRRRRGGGC               | 35        |
| RRRRRRGGGC               | 30        |
| RRRRRRGGGC               | 25        |
| RRRRRGGGC                | 25        |
| RRRRGGC                  | 20        |
| RRGGC                    | 20        |
| RRGGC                    | 15        |
| RRGGC                    | 10        |
| RRGCG                    | 5 (Ki = 158±29 nM\(^1\)) |
| GC                       | 100       |
| R                        | 65        |

Final concentration of each compound was 20 µM in the assay mixture containing the 17-mer SNAP-25 peptide substrate. Results represent an average of three assays that was rounded to nearest 5. The 100% activity of the control (None) represents a specific activity of 3.8 µmol/min/mg. \(^1\)From Figure 1.
TABLE II: Optimization of the inhibitor peptide sequence

| Peptide   | % Activity | $K_i$ (nM) | Peptide   | % Activity | $K_i$ (nM) |
|-----------|------------|------------|-----------|------------|------------|
| None      | 100        |            | RRGF      | 5          | 358±661    |
| R         | 110        |            | RRGT      | 60         |            |
| CR        | 100        |            | RRAT      | 80         |            |
| RC        | 90         |            | QRGC      | 40         |            |
| RR        | 65         |            | WRGC      | 30         |            |
| RRC       | 65         |            | RWGC      | 50         |            |
| RRG       | 100        |            | RYFC      | 80         |            |
| RGC       | 100        |            | CRGF      | 10         |            |
| RRGC      | 5          | 158±291    | CRGC      | 30         |            |
| RRGS      | 75         |            | CRRGC     | 70         |            |
| RRGL      | 5          | 664±411    | CRRGF     | 25         |            |
| RRGLL     | 25         |            | QRATKM    | 50         |            |
| RRGAL     | 35         |            | RRATKM    | 40         |            |
| RRGI      | 10         | 786±691    | RATKML    | 120        |            |
| RRGM      | 10         | 845±1041   | CRATKML   | 40         | 18002      |

Each peptide (20 µM) was incubated with the assay mixture containing 0.8 mM 17-mer SNAP-25 peptide substrate. Results represent an average of five assays that was rounded to nearest 5. The 100% activity of the control (None) represents a specific activity of 3.8 µmol/min/mg. $K_i$ was determined by assaying the reaction at a series of five substrate concentrations containing four inhibitor concentrations. The activity results were analyzed by double reciprocal plots of reaction velocity versus substrate concentration similar to those shown in Figure 1. 1From Figure 1, 2(34)
TABLE III: Effects of DTT on the inhibitory properties of the peptides

| Peptide    | IC<sub>50</sub> (µM) +DTT | IC<sub>50</sub> (µM) -DTT |
|------------|---------------------------|--------------------------|
| RRGC       | 134.0                     | 4.3                      |
| DRRGC      | 91.4                      | 51.4                     |
| RDRRGC     | 97.7                      | 75.2                     |
| RRGDC      | 230.0                     | 20.0                     |
| RRGL       | 1.7                       | 2.2                      |
| RRGI       | 2.7                       | 3.3                      |
| RRGGM      | 2.2                       | 3.3                      |
| RRGF       | -                         | 4.9                      |
| CRATKML    | 13.3                      | 20.8                     |
| QRATKM<sup>1</sup> | -                | 132.9<sup>1</sup>       |
| RRATKM<sup>1</sup> | -                | 94.9<sup>1</sup>       |

Each peptide (20 µM) was incubated with the assay mixture containing 0.9 mM 17-mer SNAP-25 peptide substrate. IC50 values were calculated from the relation IC<sub>50</sub> = ([I]*V/V₀)/(1-V/V₀) (48). Results represent an average of five assays. <sup>1</sup>(32).
LEGENDS TO FIGURES

Figure 1. Double reciprocal plots of velocity versus substrate concentration at several fixed concentrations of GRKKRRQRRRPPQC (A), RRGC (B), RRGL (C), RRGI (D), RRGM (E), and RRGF (F). LcA was incubated for 5 min at 37°C with 1.33, 0.75, 0.44, 0.33, and 0.22 mM 17-mer SNAP-25 peptide substrate in the presence of inhibitors. The reaction products were analyzed by UPLC™ as described under EXPERIMENTAL PROCEDURES. Each data point represents an average of five assays. Inhibitor concentrations: A, △, 0 nM; ○, 250 nM; and ▼, 417 nM; B-F: △, 0 nM; ○, 200 nM; ▼, 400 nM; □, 600 nM; ▲, 800 nM, and □ 1000 nM. The curves in each figure are best described as intersecting at a common point on the y-axis (not affecting maximum velocity), characteristic of a competitive inhibition such that only Km (negative reciprocal of x-intercept in the figures) is affected (60). Ki values at individual inhibitor concentrations were determined from the relation: 

\[ \text{Ki} = \left( \frac{\text{Km} \times [I]}{\text{Km}_{\text{app}} - \text{Km}} \right) \] (60).

Figure 2: Western blot of SNAP-25 in rat brain extract (total [protein] = 20 mg/ml) after reaction with LcA in the presence of peptide inhibitors. After 30 min incubation at 37°C, the 50 ml reactions were stopped by adding equal volume of 2X SDS-load buffer and immediate heating at 95°C. Nitrocellulose membranes containing the transferred protein bands were probed with anti-SNAP-25 antibody. The two adjacent-stained bands in each panel represent full-length SNAP-25 having a molecular mass of 25 kDa (upper band), and its cleaved form (lower band) after removal of the last nine amino acid peptide by the endopeptidase action of LcA.

Figure 3: Western blot of SNAP-25 in chick motor neuron extract after reaction with LcA in the presence of peptide inhibitors. Chick neurons were exposed to the indicated concentrations of RRGC for 24 h at 37°C. The cells were then extensively washed to remove excess inhibitor, and incubated with 4 nM whole BoNT/A toxin for 2 h, 4 h and 24 h. The reactions were stopped by boiling with equal volume of 2X SDS-load buffer for 5 min. Nitrocellulose membranes containing the transferred protein bands were probed with anti-SNAP-25 antibody. The two adjacent stained bands in each panel represent full-length SNAP-25 having a molecular mass of 25 kDa (upper band), and its cleaved form (lower band) after removal of the last nine amino acid peptide by the endopeptidase action of LcA. A comparison of the western blot of VAMP-2 (probed by anti-VAMP-2 antibody) in the 24 hour sample in the lowest panel serves as a protein load control for the 24 h BoNT/A cleavage lane of SNAP-25.

Figure 4: Effect of peptide inhibitors on BoNT/A and BoNT/B protease activities inside rat (A) and mouse (B) cerebellar neurons. The rat (A) and mouse (B) neurons were treated with 250 µM and 600 µM inhibitor, respectively, described under EXPERIMENTAL PROCEDURES. Rat neuron incubation was for 24 hour and mouse neuron incubation was for 4 hours. After removal of excess inhibitor by extensive washes, the cells were treated with 1 nM whole BoNT/A for 2-24 h (A) or 100 pM BoNT/A and 1 nM BoNT/B toxin for 4h (B), and were analyzed by western blots as described in Figures 2-3. The stained bands were scanned by densitometry, and the relative intensity in each lane was calculated. The results (A) are average of three independent assays, one (western blot) of which is shown at the upper panel. The inhibitor Abs252 (61) was used as a positive control. In B, a – sign above the 1st lane stands for a control without BoNT, and a positive sign above the 2nd lane stands for a control with BoNT; the rest two lanes have BoNT plus 600 µM indicated peptide inhibitors.

Figure 5: Effects of tetrapeptide inhibitors upon N2A (A) and and BE (2)-M17 (B) cells. Cells were treated for 24 h with three concentrations (600, 60, and 6 µM) of inhibitors and then measured for the presence of ATP using a luminescence-based assay as counts per second (CPS). For a cytotoxic
positive control, treatment of cells with 6 μM SDS was included. Results are expressed as %
Untreated control cells = [(CPS treated cells/CPS untreated control cells) X 100] and are the average
± S.D. of 6 wells.

Figure 6: Effects of tetrapeptide inhibitors on phosphorylation of P38 in BE (2)-M17 cells. Cell
monolayers were treated with 6 μM (A) and 600 μM (B) of the peptides and incubated at 37°C, 5% CO₂.
After 30 and 60 min (x-axis), media was removed from the cell monolayers, washed and the
cells fixed. The presence of MAPK p38 (white bars) and phosphorylated p38 (shaded bars) were
detected using an in cell Western. PMA was included as a positive control while untreated cells
served as a negative control. Results are expressed as the average OD (450 nm) ± S.D. of 4 wells (y-
axis).
Figure 2

![Figure 2 Image]

**[Inhibitor] (μM)**

|       | 0   | 0   | 30  | 120 | 140 | 160 |
|-------|-----|-----|-----|-----|-----|-----|
| LcA, 60 nM | -   | +   | +   | +   | +   | +   |
| RRGC  |     |     |     |     |     |     |
| RRGL  |     |     |     |     |     |     |
| RRGI  |     |     |     |     |     |     |
| RRGM  |     |     |     |     |     |     |
| CRARKML |    |     |     |     |     |     |
| GRKRRQR-RRPPQC | | | | | | |

Figure 3

![Figure 3 Image]

Figure 4A

![Figure 4A Image]

Figure 4B

![Figure 4B Image]
Figure 5

![Graph A and B showing % Uninjured Cell Control for Controls and Tetrapeptides.]

Figure 6

![Graph A and B showing Absorbance at 450 nm for Controls and Tetrapeptides.]
Basic tetrapeptides as potent intracellular inhibitors of type A botulinum neurotoxin protease activity
Martha Hale, George Oyler, Subramanyam Swaminathan and S. Ashraf Ahmed

J. Biol. Chem. published online October 20, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.146464

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts