Functional Assay for Botulinum Neurotoxin Type A Utilizing the Neuronal Receptor Protein SV2c

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Abstract

Botulinum toxin progresses through a three step process leading to the interruption of synaptic transmission. The first step is binding of toxin to neuronal cells and incorporation of the toxin into an endosome; the second step is translocation of the enzymatic light chain out of the endosome and into the cytosol of the neuron; and the final step is cleavage of synaptosomal proteins to prevent vesicle docking and neurotransmitter release.

Recent biochemical and molecular genetic studies have established synaptic vesicle glycoprotein 2C (SV2c) as the protein receptor for Botulinum Neurotoxin Type A (BTA). The luminal domain loop of SV2c, between transmembrane domains 7 and 8, has been shown to be the location of BoNT/A binding (Mahrhold et al FEBS Lett. 2006). Utilizing recombinant GST-SV2c, we have shown through pull-down experiments that we can detect both the binding of BTA to SV2c and enzymatic activity using the FRET peptide SNAPtide®. We describe the reaction conditions and the detection limits using multiple FRET substrates.

With this assay we can examine two of the three steps of toxin activity, binding and cleavage. In the future, we plan to establish this as a functional assay to monitor binding and enzymatic activity of Botulinum Neurotoxin Type A in order to demonstrate a direct correlation to the gold standard, the mouse bioassay.

Introduction

Multiple assays have been developed to detect botulinum toxin based solely on antigenic properties and/or enzymatic activity. Now with the recent identification of SV2c as the protein receptor for BTA[1,2] new detection methods can be attempted that combine multiple steps of the disease process of botulinum toxin. Capture of the BTA with the SV2c receptor binding domain demonstrates the specific binding which is then detected using a FRET peptide. Cleavage of this specific BTA substrate indicates the specific endopeptidase activity of the BTA. By combining these activities this assay mimics two of the three *in vivo* functions of the toxin.

The specific binding domain of SV2c for BTA has been shown to be the luminal domain loop between transmembrane domains 7 and 8.[1,2] A diagram of the structure of SV2c is shown in **Figure 1**. We have purified the SV2c luminal binding domain as a GST fusion and utilized it in our capture assays. There are several strategies that we have attempted. We have immobilized the GST-SV2c to anti-GST antibodies that are covalently attached to Protein A coated resin. We have also bound the GST-SV2c to magnetic beads coated with glutathione (MagneGST). See **Figure 2** for an illustration of these two strategies.

Our goal for this research is to establish an assay which demonstrates binding and catalytic activity and that can compete with the mouse bioassay.

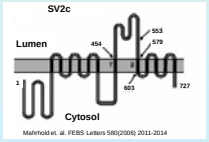

Figure 1: SV2C structure

The binding domain for Botulinum Neurotoxin Type A has been shown to be in the luminal loop, amino acids 454-579.

Materials

SNAPtide® substrate # 520 contains the FRET pair o-aminobenzoic acid/2,4 dinitrophenyl (o-Abz/Dnp) and SNAPtide® substrate Mca/Dnp contains (7-methoxy-coumarin-4-yl)acetyl/2,4 dinitrophenyl as its FRET pair. Botulinum Neurotoxin Type A (Product #130A) and the SNAPtide® substrates are products of List Biological Laboratories. Plasmid encoding the binding domain of human SV2c was provided by A. Rummel. MagneGST beads (V8611) were obtained from Promega. Immobilized Protein A (#45215) was obtained from Pierce. Monoclonal Anti-GST antibody was obtained from Abcam (ab18183).

Methods

MagneGST experiments: MagneGST beads (200µl) were mixed with 125µg of GST-SV2c and incubated for 1 hour with mixing in the presence of 1% BSA. The MagneGST beads were washed and reconstituted in 1.2 ml of 50mM HEPES, pH 8 buffer. Fifty microliters of the beads were used in each reaction. Various concentrations of Botulinum Neurotoxin Type A were added to each tube. Toxin binding was 1-4 hours at room temperature with mixing. Unbound toxin was washed away and 400µl of SNAPtide® reaction buffer (50 mM HEPES, pH 8, 5 mM DTT, 1 mM $ZnCl_2$, 0.1% BSA) and 20µM SNAPtide® (o-abz/Dnp) #520 or SNAPtide ® (Mca/Dnp) was added to each tube. The reaction tubes were mixed at 37ºC for thirty minutes and then overnight at room temperature, unless otherwise noted. They were then placed on a magnetic stand and reaction mixture was pipetted from the tubes. The beads were washed with 50mM HEPES buffer and 250µl of reaction and wash were placed into a single well of a black 96 well plate. The plate was read using a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices). The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the o-Abz/Dnp substrate and 325nm /398nm for the Mca/Dnp substrate.

Immobilized Protein A experiments: Immobilized Protein G resin (0.2ml) was washed and crosslinked to 50µg of Anti-GST antibody (Abcam 18183) using the the Seize X Protein A Immunoprecipitation kit from Pierce. After crosslinking, the resin was washed using the Gentle Ag/Ab binding and elution buffers from Pierce (#2102). GST-human SV2c (125 µg) was added to the protein A resin with Anti-GST antibodies and incubated overnight at 4ºC. The bound resin was washed and 100µl was aliquoted into each spin column. Dilutions of BTA were added to each column and incubated for 4 hours at room temperature. Unbound toxin was washed away and SNAPtide® reaction buffer (50 mM HEPES, pH 8, 5 mM DTT, 1 mM $ZnCl_2$, 0.2% Tween-20) and 20µM SNAPtide® (o-Abz/Dnp) #520 was added to each column. The reactions were mixed at 37ºC for thirty minutes and then overnight at room temperature. The reaction mixtures were centrifuged from the columns and placed into a black 96 well plate. The resin was washed several times with 50mM HEPES buffer and added to the wells. The plate was read using a SPECTRAmax GEMINI XS fluorescence microplate reader (Molecular Devices). The excitation and emission wavelengths were set to 321 nm and 418 nm, respectively, for the o-Abz/Dnp-substrate

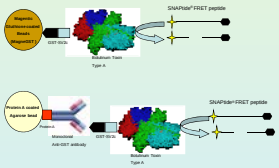

Figure 2: Capture Methods. Schematics showing the use of SV2c in the binding and detection of Botulinum Neurotoxin Type A.

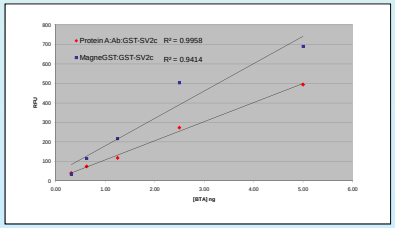

Figure 3: Comparison of MagneGST and Protein A capture assays using GST-SV2c. Both assays detect low amounts of Botulinum Neurotoxin. The MagneGST assay is a simpler, more cost effective method and can achieve higher signal to noise ratios.

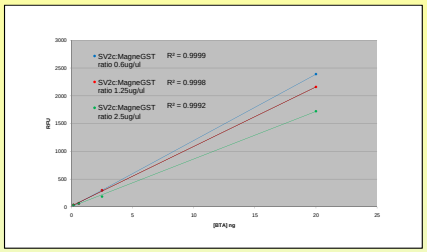

Figure 4: SV2c to MagneGST bead ratio. Several different ratios of SV2c to beads were tested. The low ratio (0.6µg/µl) was 125µg SV2c:200µl beads. The middle ratio (1.25µg/µl) was 250µg SV2c:200µl beads. The high ratio (2.5µg/µl) was 500µg SV2c:200µl beads. At the lower concentrations of toxin the difference between the ratios was quite small. The difference becomesnoticeable as the toxin concentration reaches 5 and 20ng.

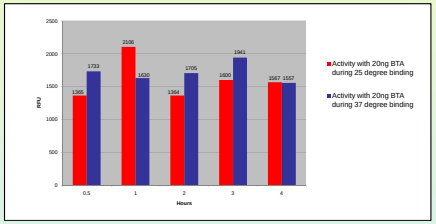

Figure 5: Toxin exposure time at 25 and 37ºC. The duration and temperature of the toxin binding step does not significantly alter the amount of enzymatic activity detected. Therefore, in an effort to shorten the assay time 1hour binding at room temperature was chosen. Following the toxin binding, these assays were carried out using a 30 minute 37°C reduction step followed by room temperature overnight incubation.

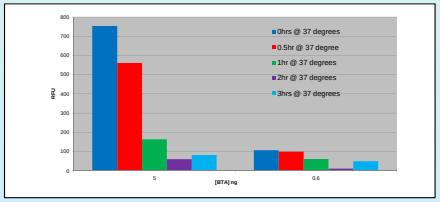

Figure 6: 37ºC versus 25ºC reduction step. The requirement of a 37°C incubation, prior to room temperature overnight incubation of bound toxin with reaction buffer, was tested. It was thought that incubation at 37°C would aid in the reduction of the toxin. However, as the incubation at 37°C is extended, the loss of detectable activity is more apparent. This effect is larger for 5ng than 0.6ng of BTA.

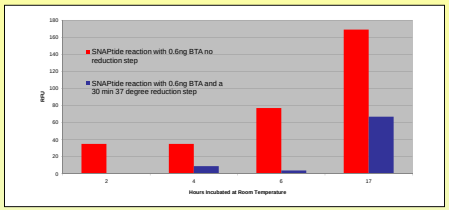

Figure 7: Optimization of reaction time. The FRET peptide cleavage was monitored for a series of time points in order to determine the optimal reaction time. Enzymatic activity with 0.6ng of BTA was clearly detected after 6 hours in the absence of a 30 minutes reduction incubation at 37°C. When the 37°C reduction step was included, enzymatic activity was only detected with 0.6ng of BTA after overnight incubation.

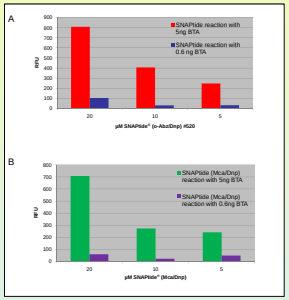

Figure 8: Test of different substrate concentrations. Two different FRET peptides were tested at three different concentrations using two different BTA concentrations in the MagneGST capture assay. These assays had no 37°C reduction step and were overnight reactions. No inhibitory affects were observed using 20µM substrate.

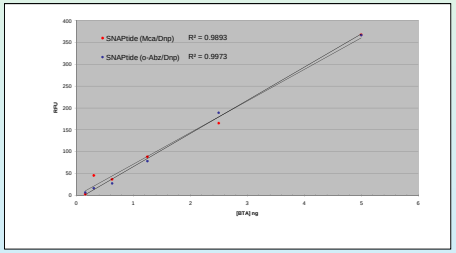

Figure 9: Botulinum Neurotoxin Type A titration using both SNAPtide® (o-Abz/Dnp) #520 and SNAPtide® (Mca/Dnp). These assays were run with one hour toxin binding at room temperature and a six hour room temperature cleavage reaction. Both substrates show a linear response to the toxin concentrations. Three hundred picograms of BTA can be detected with either peptide.

References:

1) Mahrold et al. FEBS Letters. 2006 April 3, 580(8):2011-4.

2) Dong et al. Science. 2006 April 28; 312(5773):592-6