Botulinum Neurotoxin A Changes Conformation upon Binding to Ganglioside GT1b[†]

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ABSTRACT: In this work, the kinetics of the binding of botulinum neurotoxin A (BoNT/A) to ganglioside GT1b were studied using surface plasmon resonance (SPR). The neurotoxin bound polysialylated gangliosides, and that binding was affected by the ionic strength of the buffer. Although the level of binding was decreased at higher ionic strengths, it could be easily observed in Tris buffer, containing 150 mM NaCl. Data analysis revealed that the binding of BoNT/A to a GT1b-containing phospholipid monolayer did not fit a traditional 1:1 model. Subsequent studies, in which the time of contact between BoNT/A and GT1b was varied, indicated that the BoNT/A−GT1b complex became more stable over time, as evidenced by its reduced rate of dissociation. Circular dichroism indicated that when BoNT/A was incubated with GT1b, it underwent a conformational change that resulted in an increase in α-helix content and a decrease in *β*-sheet content. Therefore, the SPR kinetic data were fit to a conformational change model and kinetic rate constants determined. The apparent K_D values obtained for the binding of BoNT/A to ganglioside GT1b ranged from 2.83×10^{-7} to 1.86×10^{-7} M, depending on the ionic strength of the buffer.

Botulinum neurotoxin is the deadliest of all known biological substances, being 100 billion times more deadly than cyanide and ∼1 million times more poisonous than cobra toxin (*1*). There are seven different serotypes of botulinum neurotoxin (BoNT), designated by the letters A−G (BoNT/A−G).[1] These serotypes, along with tetanus neurotoxin (TeNT), make up the clostridial neurotoxin family. Produced as a single-polypeptide chain by the bacterium, botulinum neurotoxin (150 kDa) is subsequently cleaved into two functional subunits, held together by a disulfide bond. The 100 kDa "heavy chain" is made up of a C-terminal half (H_C or C-fragment), which mediates cellular adherence, and an N-terminal half (H_N), believed to mediate the cytoplasmic entry of the light chain (*2*). The 50 kDa "light chain" is the active subunit of the neurotoxin, a zinc endoprotease, which catalyzes the cleavage of one of the SNARE proteins at the neuromuscular junction. By cleaving a SNARE protein and preventing fusion of synaptic vesicles with the presynaptic terminal, BoNT induces a flaccid paralysis (botulism), which can be fatal if untreated (*3*).

Although only a few hundred cases of botulism are reported each year in the United States (*4*), there is growing interest in BoNT that can be attributed to its potential for use in biological warfare (*5*). In contrast to its potential for use as a biological weapon, the potency and specificity of action of botulinum neurotoxin allow it to be used clinically to treat an ever-growing number of muscular disorders, including several dystonias, cerebral palsy, Parkinson's, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and migraines (*6*).

More than 30 years ago, polysialylated gangliosides were first mentioned as potential receptors for botulinum neurotoxin A (BoNT/A) (*7*, *8*). Gangliosides are glycosphingolipids found on the outer leaflet of cell membranes and are characterized by the presence of sialic acid. Specifically, polysialylated gangliosides such as GT1b [nomenclature according to Svennerholm (*9*)] have been shown to inhibit binding of BoNT/A to synaptosomes (*10*) and to quench BoNT/A fluorescence (*11*). BoNT/A has also been shown to directly bind gangliosides immobilized on either a thin layer chromatogram (*12*) or microtiter wells (*13*). At the cellular level, the requirement for a sialylated component (*14*, *15*) led to experiments that indicated that specific gangliosides were necessary for BoNT/A activity (*16*, *17*). *In vivo* studies using GM2/GD2-synthase knockout mice indicated that gangliosides mediate the effects of BoNT/A (*18*, *19*). Admittedly, these studies do not preclude the hypothesis that the BoNT/A receptor is comprised of both a protein and a ganglioside (*20*). However, a recent study indicated that the leading protein receptor candidate, synaptotagmin, is not involved in BoNT/A activity (*21*).

Therefore, the only well-characterized cell membrane components known to be required for BoNT/A activity are polysialylated gangliosides. While the fact that BoNT/A needs gangliosides, such as GT1b, for activity has been well documented, the kinetics of that binding have not. To observe real-time binding of BoNT/A to a ganglioside-containing phospholipid monolayer, surface plasmon resonance (SPR) was used. This technique has several advantages over

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[1] Abbreviations: BoNT/A, botulinum neurotoxin A; GT1b, trisialoganglioside GT1b; GM1, monosialoganglioside GM1; GD1a, disialoganglioside GD1a; SPR, surface plasmon resonance; CD, circular dichroism; DMPC, dimyristoyl L-α-phosphatidylcholine.

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traditional methods. It allows measurement of kinetic rate constants in the absence of added labels, such as a fluorescent probe or radioisotope, and it requires relatively small samples. SPR requires one component (ligand) to be immobilized on a sensor chip, while the other component (analyte) flows freely in solution over the surface of the chip. Since association and dissociation of the analyte are observed in real time, both on-rates (k_a) and off-rates (k_d) can be determined, thus describing the affinity of the interaction.

Our results indicate that the binding of BoNT/A to GT1b, in a phospholipid monolayer, was dependent upon ionic strength and was observed at NaCl concentrations as high as 150 mM. The kinetics of the binding were found to be complex and did not follow a simple 1:1 binding model. Additional SPR experiments showed that the BoNT/A−GT1b complex became more stable as contact time increased, indicating a possible conformational change in the neurotoxin. This hypothesis was tested by circular dichroism studies, which indicated that, upon incubation with GT1b, the conformation of the neurotoxin changed over time. Specifically, an increase in α-helix content and a decrease in β-sheet content were observed as the incubation time for BoNT/A and GT1b was increased. On the basis of these observations, a two-state conformational change model was used to fit the SPR kinetic data. The apparent K_D values that were obtained ranged from 2.83×10^{-7} to 1.86×10^{-7} M, depending on the ionic strength of the buffer.

EXPERIMENTAL PROCEDURES

Materials. DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) was purchased from Sigma (St. Louis, MO). Gangliosides GM1, GD1a, and GT1b (>98% pure) were purchased from Matreya, Inc. (State College, PA). BoNT/A was kindly provided by B. R. DasGupta (University of Wisconsin, Madison, WI). BoNT/A concentrations (milligrams per milliliter) were determined using the calculation absorbance at 280 nm/extinction coefficient of 1.63. SPR experiments were performed at 25 °C using a BIACORE 3000 instrument (Biacore, Inc., Uppsala, Sweden). HBS-N buffer [10 mM HEPES and 150 mM NaCl (pH 7.4)] was either purchased from Biacore, Inc., or freshly prepared and was used for all liposome immobilizations. Tris buffer [10 mM Tris-HCl (pH 7.4)], containing the indicated sodium chloride concentration, was used as the running buffer for the instrument as well as the sample buffer for all BoNT/A binding experiments, and was prepared immediately prior to use.

Preparation of Liposomes for SPR. DMPC (0.5 mg) in $CHCl_3$ was mixed with 2 or 5 mol % GM1, GD1a, or GT1b in a $CHCl_3$/MeOH mixture (1:1 by volume) and dried to a thin film under N_2. HBS-N (330 μL) was then added to the dried lipids and the mixture hydrated for 15 min at 37 °C with occasional vortexing to yield a 2 mM solution with respect to DMPC. The suspension was sonicated for 2−3 min and extruded through a 50 nm filter 19 times. Note that for use as a negative control, liposomes were also prepared from DMPC alone. Liposomes were used immediately following preparation.

Immobilization of Liposomes on an HPA Chip. Following a 5 min injection of 40 mM octyl glucoside at a flow rate of 5 μL/min to wash the surface of the chip, the flow rate was reduced to 2 μL/min and freshly prepared liposomes were injected for 20−30 min. This resulted in an increase in baseline of 1000−2000 RU. To create a monolayer, 20 μL of 50 mM NaOH was injected at a flow rate of 100 μL/min. Finally, nonspecific binding sites were blocked by injecting BSA (0.1 mg/mL) over the lipid monolayer for 5 min at a flow rate of 10 μL/min. Interaction of BSA with the surface of the chip did not result in a change of more than 100 RU.

Binding of BoNT/A to Immobilized Gangliosides. Liposomes comprised of DMPC alone and of DMPC containing either 2 or 5% GM1, GD1a, or GT1b were immobilized on flow cells 1−4, respectively, of an HPA chip. The system was primed three consecutive times with Tris buffer containing 50 mM NaCl. BoNT/A (50 nM) was then injected for 2 min at 10 μL/min over flow cells 1−4. Following a 5 min dissociation in buffer, residual BoNT/A bound to the chip was removed by a 2 min injection of 1 M NaCl at a flow rate of 30 μL/min. This regeneration step prepared the surface for use in subsequent cycles of BoNT/A binding. Sensorgrams of nonspecific binding to DMPC alone (flow cell 1) were subtracted from those of binding to each of the DMPC monolayers containing ganglioside (flow cells 2−4).

Kinetic Analysis of the Binding of BoNT/A to GT1b. To reduce the density of GT1b in the monolayer, these analyses were carried out using liposomes containing DMPC alone (control) or 2% GT1b in DMPC (GT1b/DMPC) immobilized on flow cell 1 and 2, respectively, of an HPA chip. The system was then primed three times with the appropriate buffer. To prepare the surface for analysis, at least 20 cycles of binding of BoNT/A (50 nM) and regeneration with 1 M NaCl were performed as described above. The BoNT/A solution was freshly prepared from an ammonium sulfate precipitate and its concentration verified by spectrophotometry, as described previously. Various concentrations of BoNT/A (500−0.24 nM) in Tris buffer, containing either 75, 100, or 150 mM NaCl, were injected (in duplicate) in random order at a flow rate of 30 μL/min over both flow cells. Following a 5 min dissociation phase, 1 M NaCl was injected for 2 min at a flow rate of 30 μL/min to remove any remaining bound BoNT/A and to prepare the surface for the next cycle. Sensorgrams from flow cell 1 (DMPC alone) were subtracted from those of flow cell 2 (GT1b/DMPC). In addition, a sensorgram of an injection of buffer alone was also subtracted from those from flow cell 2 prior to analysis. Resultant sensorgrams were analyzed using BiaEvaluation 4.1 and fit to either the 1:1 (Langmuir) model or the conformational change model, described by the following equation:

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} AB^*$$

where $K_A = (k_{a1}/k_{d1})(1 + k_{a2}/k_{d2})$ and $K_D = 1/K_A$. In this model, the analyte (A) binds to the ligand (B) to form an initial complex (AB). The complex then undergoes a change in conformation to form a more stable complex (AB*).

To determine whether the observed binding was limited by mass transfer, BoNT/A (25 nM) was injected for 2 min over the GT1b/DMPC monolayer at each of three different flow rates (5, 15, and 75 μL/min). The association phases were compared to determine whether an increase in flow rate correlated with an increase in the level of binding (data not shown). Although an increase in the extent of association

was observed upon increasing the flow rate from 5 to 15 μL/min, no significant change in association was observed when the flow rate was increased from 15 to 75 μL/min. Since kinetic experiments were performed at a flow rate of 30 μL/min, mass transfer limitations were unlikely.

Analysis of the Stability of the BoNT/A−GT1b Complex over Time. These studies were performed on the same GT1b/DMPC monolayer using the same Tris buffers used for the kinetic analyses. BoNT/A (100 nM) was injected at a flow rate of 10 μL/min over the control surface (DMPC alone) and the GT1b/DMPC monolayer for time periods that ranged from 1 to 30 min. Following the injection, buffer was allowed to flow over the surface of the chip and the dissociation of BoNT/A from GT1b was monitored for 5 min. To remove all BoNT/A to prepare the surface for the next cycle, 1 M NaCl was injected for 2 min at a flow rate of 30 μL/min. The level of nonspecific binding of BoNT/A to the DMPC monolayer was subtracted from its level of binding to the monolayer containing 2% GT1b. Due to an increase in the amount of BoNT/A bound as the contact time increased, all sensorgrams were normalized to 100 RU and aligned at the injection stop point for analysis.

Circular Dichroism Analysis of BoNT/A Incubated with GT1b. CD studies were performed using a Jasco J-710 spectropolarimeter. The secondary structure of the neurotoxin in the far-UV portion of the spectrum (190−250 nm) was monitored using a 0.1 mm cell at 25 °C. BoNT/A (0.67 μM) alone and a mixture of BoNT/A (0.67 μM) and GM1 or GT1b (134 μM) were prepared in Tris buffer. Spectra (in triplicate) were acquired for the mixture of BoNT/A and GT1b at 2, 10, 30, 90, and 180 min and for the mixture of BoNT/A and GM1 at 2 and 180 min. To determine whether the spectra of ganglioside in buffer or of buffer alone changed over time, spectra of each were collected prior to and immediately following those of BoNT/A incubated with ganglioside. These were then subtracted from the corresponding spectra of BoNT/A with ganglioside. The resultant spectra were compared to those obtained for BoNT/A alone with the spectra of buffer alone subtracted. To determine the overall change in secondary structure, CD spectra were fit using the program JFIT, developed by Bernhard Rupp at the Lawrence Livermore National Laboratory (Livermore, CA), in 1997.

RESULTS

Binding of BoNT/A to Immobilized Gangliosides. As discussed previously, BoNT/A has been shown to preferentially interact with polysialylated gangliosides. However, the kinetics of this binding have not been well characterized. To determine whether SPR could be used to monitor the kinetics of this interaction, the binding of BoNT/A to mono (GM1)-, di (GD1a)-, or trisialogangliosides (GT1b) was studied. Injection of BoNT/A (50 nM) at a flow rate of 10 μL/min for 2 min over DMPC monolayers containing 2% GM1, GD1a, or GT1b yielded the sensorgrams shown in Figure 1. The data indicate that the neurotoxin binds gangliosides in a specific manner (GT1b > GD1a ≫ GM1), which agrees with data obtained using ELISAs (*13*). Therefore, it was concluded that this SPR system can be used to monitor the specific binding of BoNT/A to polysialylated gangliosides. Since GT1b was the preferred ganglioside ligand for BoNT/A, it was the ligand used in subsequent studies.

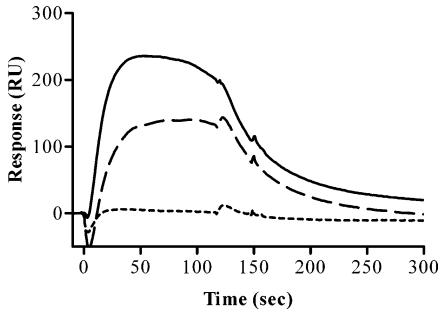

FIGURE 1: Sensorgrams obtained for the binding of BoNT/A (50 nM) to a DMPC monolayer, containing 2% GT1b (—), GD1a (− − −), or GM1 (- - -).

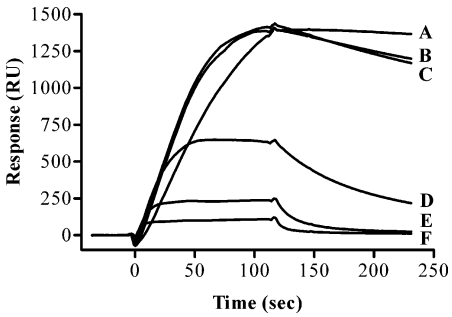

FIGURE 2: Sensorgrams obtained for the binding of BoNT/A (50 nM) to a DMPC monolayer, containing 5% GT1b in Tris buffers at different ionic strengths. The concentration of NaCl in the buffer was (A) 10, (B) 20, (C) 30, (D) 50, (E) 75, or (F) 100 mM.

Effect of Ionic Strength on Binding of BoNT/A to GT1b. It is well-known that the *in vitro* interaction of BoNT/A with gangliosides is dependent upon the ionic strength of the buffer (*11*−*13*). Therefore, the effect of ionic strength on the binding of BoNT/A to the GT1b/DMPC monolayer was ascertained. Buffers consisted of 10, 20, 30, 50, 75, or 100 mM NaCl in 10 mM Tris-HCl (pH 7.4). Each was used as both the running buffer for the experiment and the sample buffer for the BoNT/A. Injections (2 min) of BoNT/A (50 nM) in each of the buffers yielded the sensorgrams shown in Figure 2. The results indicate that as the ionic strength increased the observed amount of BoNT/A bound to the GT1b/DMPC monolayer decreased.

Kinetic Analysis of the Binding of BoNT/A to GT1b. To determine the overall affinity of the observed binding of BoNT/A for GT1b, as well as the association and dissociation rates, kinetic analyses were performed. Although the observed BoNT/A binding was stronger in low-ionic strength buffers (<75 mM NaCl), the lower ionic strength permitted nonspecific binding of BoNT/A to the control surface (DMPC). Therefore, to eliminate any complications caused by nonspecific binding, only buffers containing ≥75 mM NaCl were used for kinetic studies. To account for refractive index changes due to the sample injection, as well as nonspecific binding, sensorgrams of the binding of BoNT/A to the control surface (DMPC alone) were subtracted from those obtained for its binding to the GT1b/DMPC surface. Furthermore, changes in the sensorgram due to the buffer were eliminated by subtracting the sensorgram for an injection of buffer alone. Subtracted data were then analyzed using BiaEvaluation 4.1.

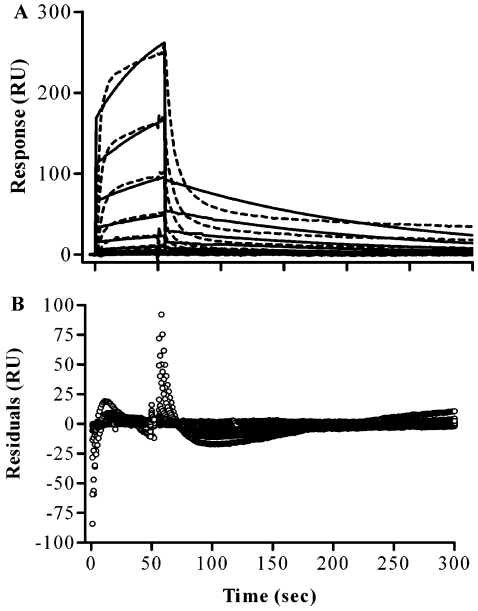

FIGURE 3: (A) Sensorgrams (- - -) obtained for the binding of different concentrations of BoNT/A (500−0.24 nM) in 10 mM Tris-HCl and 150 mM NaCl (pH 7.4) to a 2% GT1b/DMPC monolayer, fit to the 1:1 Langmuir binding model (solid lines). (B) Plot of the residuals (difference between the data and the fit).

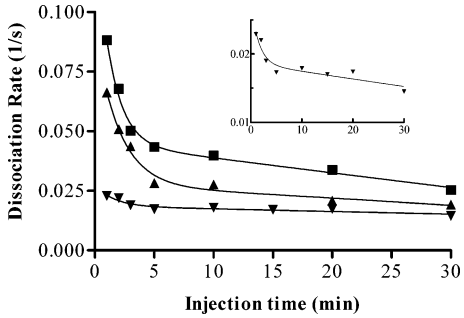

FIGURE 4: Plot of the injection time of BoNT/A (100 nM) in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (■), 100 mM NaCl (▲), or 75 mM NaCl (▼) over the 2% GT1b/DMPC surface vs dissociation rate. (The inset shows the values obtained for BoNT/A in buffer, containing 75 mM NaCl, plotted on a graph with an expanded *Y*-axis.) Dissociation rates were obtained by fitting an initial dissociation phase (10 to 20 s following the end of the injection) using a simple Langmuir dissociation model. Curves were generated using nonlinear regression (curve fit) using GraphPad Prism version 3.0.

The kinetic data that were obtained were not fit by a 1:1 (Langmuir) binding model (see Figure 3), as indicated by the large differences (residuals) between the data and the model. Therefore, to determine the reason for the deviation from 1:1 binding, additional experiments were performed. A recent report indicates that BoNT/A has only one binding site for GT1b (*22*). Therefore, it is unlikely that the observed deviation from a simple 1:1 binding model was due to multiple binding events. However, to distinguish experimentally between multiple binding sites and possible changes in the neurotoxin upon binding, the time of contact between BoNT/A and GT1b was varied. Analysis of the dissociation phases obtained after injection of BoNT/A for various times over GT1b indicated that as the contact time increased, the initial rate of dissociation decreased (Figure 4). This indicated that the binding was multiphasic, and the BoNT/A−GT1b complex became more stable over time. To determine

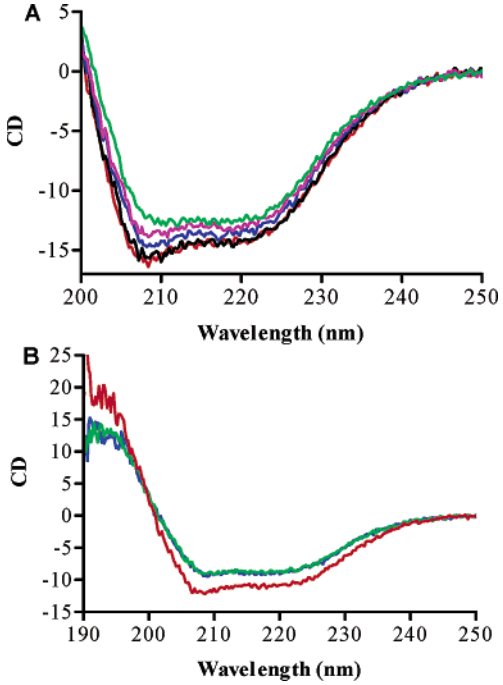

FIGURE 5: CD spectra obtained for BoNT/A and gangliosides. (A) BoNT/A (0.67 μM) alone (green) or incubated with GT1b (137 μM), in 10 mM Tris-HCl (pH 7.4) for 10 (purple), 30 (blue), 90 (black), or 180 min (red). (B) BoNT/A (0.67 μM) alone (green) or incubated with 137 μM GM1 (blue) or 137 μM GT1b (red) for 180 min.

whether this reflected a change in conformation, CD spectra were obtained for BoNT/A alone and after exposure to GT1b for increasing periods of time.

CD Studies of the Interaction of BoNT/A with GT1b. Spectra were collected for buffer, BoNT/A, GT1b, and BoNT/A incubated with GT1b for various time intervals (from 2 to 180 min). Following subtraction of the spectra obtained for GT1b and buffer alone, the spectra obtained for BoNT/A incubated with GT1b were compared to that obtained for BoNT/A alone. As shown in Figure 5A, the intensity of the spectra for BoNT/A increased during the first 90 min that it was incubated with GT1b. Essentially no change in spectra was observed over the next 90 min. This change in CD spectra indicated that the toxin underwent a conformational change, as predicted by the SPR data.

To determine whether the observed change in spectra was specific to ganglioside GT1b, or simply the effect of a lipid environment on the toxin, additional CD spectra were obtained. Since SPR data indicated that BoNT/A did not bind to ganglioside GM1, it was chosen as a nonbinding, control lipid for the CD experiments. Spectra were collected for BoNT/A alone, or BoNT/A incubated with either GM1 or GT1b (137 μM) for 180 min. As shown in Figure 5B, incubation with GM1 had no effect on the CD spectra of BoNT/A, whereas a clear increase in spectra was observed for BoNT/A when it was incubated with GT1b. Fitting of the CD spectra (190−250 nm) using the program JFIT indicated a significant increase in α-helical content from 32 $\pm$ 1.4% (BoNT/A) to 57 $\pm$ 2.5% (BoNT/A with GT1b) and a significant decrease in β-sheet content from 40 $\pm$ 1.8% (BoNT/A) to 17 $\pm$ 0.7% (BoNT/A with GT1b). As the fitting of CD data is often problematic, the absolute numbers should be considered estimates. However, the results reported here

Table 1: Kinetic Constants Obtained from the Fit to the Conformational Change Model of Sensorgrams Obtained for the Binding of Different Concentrations of BoNT/A (500−0.24 nM) to a 2% GT1b/DMPC Monolayer

[NaCl][a] (mM)	k_{a1} (M^{-1} s^{-1})	k_{d1} (s^{-1})	k_{a2} (s^{-1})	k_{d2} (s^{-1})	R_{max} (RU)	K_D (M)	χ^2
150	1.49×10^5	0.125	5.44×10^{-3}	2.78×10^{-3}	558	2.83×10^{-7}	6.59
100	3.75×10^5	0.0995	1.83×10^{-3}	5.14×10^{-3}	2020	1.95×10^{-7}	8.33
75	3.38×10^5	0.0879	1.29×10^{-3}	3.22×10^{-3}	1970	1.86×10^{-7}	10.6

[a] The concentration of NaCl in 10 mM Tris-HCl (pH 7.4).

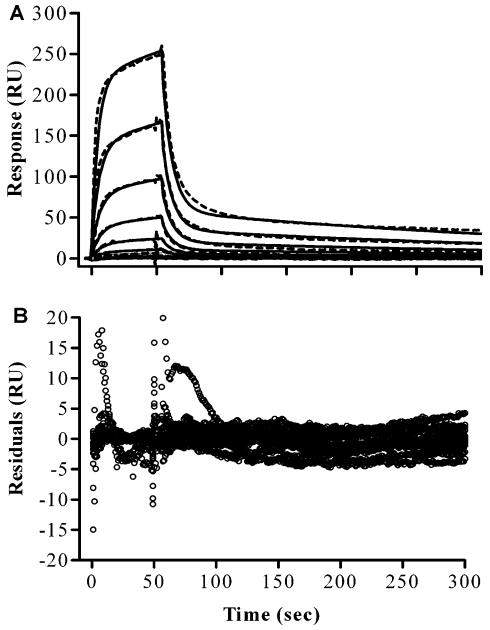

FIGURE 6: (A) Sensorgrams (- - -) obtained for the binding of different concentrations of BoNT/A (500−0.24 nM) in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl to a 2% GT1b/DMPC monolayer. Data were fit to the two-state conformational change model (—). (B) Plot of the residuals (difference between the data and the fit).

for BoNT/A are in good agreement with those from previously reported CD studies (27 ± 2% α-helix and 36 ± 1% β-sheet) (*23*).

Analysis of SPR Kinetic Data. The observation that the BoNT/A−GT1b complex became more stable as contact time was increased, coupled with the observed change in toxin structure found by CD, provided support for fitting the kinetic data to a two-state, conformational change model. The fits of the data to the model, including residuals, are shown in Figure 6 and in the Supporting Information. Kinetic constants obtained from these fits are listed in Table 1. Apparent K_D values ranged from 2.83×10^{-7} to 1.86×10^{-7} M. Measures of the goodness of fit of the model (χ^2) ranged from 6.59 to 10.6, which were less than 2% of the R_{MAX} (maximal binding). Typically, χ^2 values of less than 5% of R_{MAX} are considered acceptable. Admittedly, the maximum binding capacity of the surface (R_{MAX}) is somewhat greater than ideal for kinetic analysis, especially at the lower ionic strengths. However, to compare the binding kinetics for each of the ionic strengths without additional variables, the mole percentage of GT1b was kept constant at 2%. In addition, control experiments were performed at each of the ionic strengths to evaluate mass transfer limitations, as described above.

DISCUSSION

SPR has been used to determine the binding kinetics of other toxin−ganglioside interactions, including the binding of the C-fragment of tetanus toxin to GT1b (TeNT-H_C) (*24*, *25*). Fitting of that data to a simple 1:1 model gave a K_D of 10^{-7} M. However, subsequent studies indicated that TeNT-H_C had more than one binding site for GT1b. Analysis of the crystal structure of TeNT-H_C complexed with a synthetic GT1b analogue indicated that there were two binding sites in the β-trefoil domain (*26*). More recent mutagenesis studies confirmed the presence of two ganglioside binding sites in TeNT, and SPR analysis indicated that the binding of TeNT-H_C to GT1b was complex and did not follow a 1:1 model (*27*). Considering that tetanus neurotoxin and BoNT/A are both members of the clostridial neurotoxin family and their amino acid sequences are 52% similar (34% identical) in the carboxy-terminal half of their heavy chains (binding domains), it was hypothesized that the interaction of BoNT/A with GT1b would be similar to that reported for tetanus. As anticipated, the observed kinetics for the binding of BoNT/A to GT1b were complex and did not follow a 1:1 model. It was hypothesized that the observed deviation from simple 1:1 kinetics might be due to either mass transfer limitations, multiple binding sites, or a change in the toxin upon binding to GT1b.

To observe whether there were mass transfer limitations, BoNT/A was injected over the GT1b/DMPC monolayer at different flow rates and the association phases were compared. Although binding was limited by mass transfer at slower flow rates, it was concluded that binding of BoNT/A to GT1b was not limited by mass transfer at the flow rate used for kinetic experiments (30 μL/min).

Several published observations indicated that the observed deviation from a 1:1 binding model was probably not due to multiple binding sites. For example, comparison of the sequence of BoNT/A to the crystal structure of TeNT-Hc led to the prediction that BoNT/A contained only a single binding site for GT1b (*26*, *28*). Furthermore, results of studies in which residues in the predicted binding site were mutated coupled with those obtained from mass spectroscopy indicated that BoNT/A had only one binding site for GT1b (*22*). Therefore, while it was considered unlikely that the poor fit to the 1:1 model reflected multiple sites, the question was addressed further by monitoring the effect of contact time on the rate of dissociation of BoNT/A from GT1b. By injecting BoNT/A for various times over GT1b/DMPC, and analyzing the dissociation phases, we observed that as contact time increased, the initial rate of dissociation decreased. These results indicated that the BoNT/A−GT1b complex became more stable with time, and that the deviation from the 1:1 binding model could be attributed to that fact. Similar studies have been performed previously and determined to be effective at elucidating the mechanisms underlying complex binding kinetics (*29*, *30*).

These observations led to the hypothesis that a change in conformation occurred when BoNT/A bound GT1b. CD

studies were performed to test this hypothesis. Using a 200-fold molar excess of GT1b, an increase in the intensity of the CD spectra for BoNT/A was observed. In agreement with the SPR data, the change seen in the CD spectra over time indicated a change in the secondary structure of the neurotoxin. Fitting of the data indicated a 25% increase in α-helix content and a 23% decrease in β-sheet content upon incubation of BoNT/A with GT1b for 3 h. These results were consistent with those published for TeNT, indicating a similar change in CD spectra upon binding to GT1b (*31*, *32*).

On the basis of these results, the SPR kinetic data were fit to the conformational change model provided in BiaEvaluation 4.1, and were found to fit the model well. Residuals and χ^2 values fell within the acceptable range for Biacore analyses, which was not the case when the data were fit to the 1:1 (Langmuir) model. Apparent K_D values obtained for the binding of BoNT/A to the GT1b/DMPC monolayer using the conformational change model were comparable to those reported for TeNT-H_C (10^{-7} M). The results obtained for BoNT/A also agree relatively well with the apparent K_D of 2.3×10^{-8} M (*33*), obtained in equilibrium binding studies of the adherence of BoNT/A-H_C to GT1b-containing liposomes. Admittedly, the lipid monolayer used in this SPR study does not represent the composition of a neuronal membrane. However, it is important to note that similar affinities were observed in studies of the binding of [^{125}I]-BoNT/A to cholinergic synaptosomes and presynaptic plasma membranes, where "low-affinity" binding sites were determined to have K_D values of approximately 2.6×10^{-8} and 3.0×10^{-8} M, respectively (*34*).

It has been hypothesized that BoNT/A binds to two receptors, where the first, lower-affinity, binding is to ganglioside and the second, higher-affinity, binding is to a protein (*20*, *35*). This model appears to hold true for BoNT/B, where synaptotagmins I and II, along with gangliosides, have been identified as coreceptors for the toxin (*21*, *36*−*38*). However, in contrast to BoNT/B, Dong et al. (*21*) recently reported that synaptotagmins are not involved in the binding and uptake of BoNT/A. Therefore, the aim of this work was to study the kinetics of binding of BoNT/A to its only known receptor, ganglioside GT1b, in a phospholipid environment. Interestingly, the results reported here are not inconsistent with the hypothesis that BoNT/A binds to more than one receptor. Perhaps the observed conformational change in BoNT/A, seen upon its binding to GT1b, allows the neurotoxin to subsequently bind to a second receptor, such as a protein. If a coreceptor can be identified, it could be incorporated into the model membrane described here, and binding kinetics could be studied.

The interaction of BoNT/A with GT1b has been found to be dependent on ionic strength, when monitored by an ELISA (*13*), fluorescence (*11*), or TLC overlay (*12*). Studies monitoring the binding of the neurotoxin to synaptosomes (*39*) or cultured murine spinal cord neurons (*14*) also indicated that it was dependent on ionic strength. Therefore, the observation that the extent of binding of BoNT/A to GT1b/DMPC monolayers decreased as the ionic strength of the buffer increased was expected. However, using SPR, the binding of BoNT/A to GT1b could be observed at NaCl concentrations as high as 0.15 M. The affinity of the neurotoxin for GT1b appeared to be relatively strong, and the toxin−ganglioside complex appeared to become more stable over time. All of these observations provide support for the hypothesis that cell surface gangliosides could serve as receptors for BoNT/A.

In conclusion, the results described here indicate that SPR is an effective method for quickly and efficiently monitoring real-time binding of BoNT/A to ganglioside-containing phospholipid monolayers. It can be used to monitor the effect of changes in physical conditions on binding, and to characterize the binding of other BoNT serotypes or preparations of BoNT subunits to their putative cell surface receptors. In addition, SPR could also be used to efficiently test antibodies or potential antagonists for their ability to inhibit the binding of botulinum neurotoxin to ganglioside-containing monolayers.

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SUPPORTING INFORMATION AVAILABLE

Sensorgrams of the binding of BoNT/A in 10 mM Tris buffer, containing either 75 or 100 mM NaCl, to 2% GT1b, fit to the conformational change model and of the effect of contact time of BoNT/A on its dissociation from the 2% GT1b/DMPC monolayer. This material is available free of charge via the Internet at http://pubs.acs.org.

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