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The receptor binding domain of botulinum neurotoxin serotype C binds phosphoinositides

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Abstract

Botulinum neurotoxins (BoNTs) are the most toxic proteins known for humans and animals with an extremely low LD₅₀ of ~ 1 ng/kg. BoNTs generally require a protein and a ganglioside on the cell membrane surface for binding, which is known as a “dual receptor” mechanism for host intoxication. Recent studies have suggested that in addition to gangliosides, other membrane lipids such as phosphoinositides may be involved in the interactions with the receptor binding domain (HCR) of BoNTs for better membrane penetration. Using two independent lipid-binding assays, we tested the interactions of BoNT/C-HCR with lipids *in vitro*. BoNT/C-HCR was found to bind negatively charged phospholipids, preferentially phosphoinositides in both assays. Interactions with phosphoinositides may facilitate tighter binding between neuronal membranes and BoNT/C.

Keywords

botulinum neurotoxin; BoNT/C; botulism; phosphoinositides; membrane binding

1. Introduction

Botulinum neurotoxins (BoNTs) produced by the bacterium *Clostridium botulinum*, are considered the most toxic substance for humans and animals and cause the fatal disease botulism [1]. These neurotoxins inhibit the release of neurotransmitters from neuronal cells resulting in flaccid paralysis [2]. Because of the potent toxicity and ease of production, the Centers of Disease Control (CDC) in the United States has listed BoNTs as one of the six highest-risk “Class A” threat agents for bioterrorism [3]. There are seven related BoNT serotypes (A–G), each of which is a 150 kDa protein containing an N-terminal 50 kDa light chain (Lc) and the C-terminal 100 kDa heavy chain (Hc). The Hc can be further divided into a 50 kDa N-terminal translocation domain and a 50 kDa C-terminal receptor binding domain (HCR). The HCR domain contains two 25 kDa sub-domains: Hcn and Hcc. The Lc is a metalloprotease that specifically cleaves SNAP-25 (synaptosome associated protein of 25 kDa) and synaptobrevin [4], proteins that are essential for neurotransmission. The translocation domain is involved in the transportation of BoNTs from endosomes into cytosol, while the HCR domain is responsible for interactions with neuronal membranes.

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The mechanism underlying BoNT intoxication is relatively clear for most of the serotypes. In general, the HCR domain recognizes “dual receptors” on the neuronal cell surface, a protein and a ganglioside receptor [5]. Initial binding of BoNTs with the peripheral neuromuscular junction is mediated by the association between BoNT and complex gangliosides (polysialogangliosides). It has been shown that all seven BoNTs utilize gangliosides for membrane recognition and ganglioside-binding sites within toxins have been identified and characterized by X-ray crystallography [6–9]. The dual receptor model proposes that the gangliosides are not sufficient for the internalization of the toxin and specific protein receptors are required for the uptake into motoneurons [5]. Two major types of botulinum neurotoxin membrane protein receptors have been identified: synaptotagmin (Syt) I and II, and synaptic vesicle protein 2 (SV2). The B and G serotypes bind Syt I or II, and serotypes A, D, E, and F bind SV2 ([10] and reviewed by [11]). A protein receptor has not been identified for serotype C and it has been suggested that BoNT/C does not need a protein receptor since treating rat brain synaptosomes with proteases did not diminish toxin binding [12]. In addition to the protein and gangliosides receptors, recent studies have suggested that BoNTs also interact with other membrane surface lipids, such as phosphoinositides, possibly to help position and orientate the translocation domain for subsequent insertion into the membranes [13]. In the present study, we investigated the *in vitro* binding capacity of BoNT/C-HCR with phospholipids and demonstrated that BoNT/C-HCR binds negatively charged phosphoinositides.

2. Materials and methods

2.1 Protein expression and purification

The DNA sequence encoding the *C. botulinum* serotype C receptor binding domain (N866-E1291) was codon-optimized for expression in *Escherichia coli* (DNA 2.0; Supplementary Figure 1), synthesized with *Xho*I and *Nde*I restriction sites at the 3' and 5' ends respectively. The synthetic gene was cloned into the expression vector pJexpress411 at the *Xho*I and *Nde*I restriction sites. pJexpress 411 contains a 6×his tag in the C-terminus. Protein expression followed the protocols previously described in detail for BoNT/D-HCR and BoNT/CD-HCR [14–16]. Briefly, the *E. coli* bacterial host strain BL21 (DE3) was used and protein expression was induced at a low isopropyl- β -D-1-thiogalactopyranoside (IPTG) concentration (0.05 mM) and low temperature (12 °C). Expressed BoNT/C-HCR was initially purified on a Ni-NTA agarose column (Qiagen) following standard protocols from the manufacturer, and then further purified on a HiTrap Q ion exchange column (GE Healthcare).

2.2 Lipid dot-blot assay

Pre-spotted PIP membrane strips or arrays with phospholipids and phosphoinositides were purchased (Echelon Bioscience), and blocked using TBS-T-BSA buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, 3% fatty acid-free BSA, pH 8.0) for 1 hr at room temperature. The membrane strip was incubated with His-tagged BoNT/C-HCR at 0.5 μ g/mL at 4 °C overnight and then washed three times with TBS-T-BSA. An anti-6×His mouse monoclonal antibody (USBiological) at a 1:1,000 dilution was incubated with the membrane for 2 hrs at room temperature. After washing, the membranes were incubated with the secondary antibody, goat anti-mouse IgG (Jackson ImmunoResearch Laboratories), at a 1:10,000 dilution in TBS-T-BSA for 1 hr at room temperature. The membranes were washed again and then incubated with SuperSignal West Dura substrate (Thermo Scientific), prepared according to the manufacturer's instructions. Signal was detected using a Lumi-imager (Roche).

2.3 Liposome-binding assay

The natural phosphoinositides from Folch fraction I brain extract (a mixture of diphosphoinositide, triphosphoinositide, phosphatidylinositol and phosphatidylserine) was purchased from Sigma, and the other lipids were from Avanti Polar Lipids. Lipids were dissolved in chloroform and mixed in the respective ratios and dried under a nitrogen stream. TBS buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.0) was used to re-suspend the lipids followed by incubation at 37 °C for 1 hr. The solution was vortexed for 5 min, and then centrifuged at 20,000×g (4 °C) for 10 min. The pellets were re-suspended in 50 µL TBS with 2 mM MgCl₂ to a concentration of 2 µg/µL. Two µg of purified BoNT/C-HCR was then added to the resuspended liposomes and the solution was incubated at 30 °C for 30 min, and then centrifuged at 20,000×g (4 °C) for 10 min. Distribution of BoNT/C-HCR in the pellet and supernatant fractions was analyzed by SDS-PAGE. The intensity of the stained BoNT/C-HCR protein bands in the pellet and supernatant fractions was detected as above with a Lumi-imager and the signal was quantified with LumiAnalyst software (Roche).

3. Results and discussion

To investigate whether BoNT/C-HCR interacts with lipids, other than gangliosides, and to define which lipids it potentially binds, we utilized a protein lipid dot-blot assay. Purified His-tagged BoNT/C-HCR was incubated with a membrane pre-spotted with 15 different types of lipid molecules. After washing, bound protein was detected using an anti-His antibody. As shown in Fig. 1A, BoNT/C-HCR clearly bound phosphoinositides (PIs) with either high affinity (PI(3)P, PI(4)P, PI(5)P, and PI(3,5)P₂), or moderate affinity (PI(3,4)P₂, PI(4,5)P₂, and PI(3,4,5)P₃). Weak interactions were also observed between BoNT/C-HCR and phosphatidic acid (PA) and phosphatidylserine (PS). It is worth noting that these lipids are all negatively charged and that interactions were not detected between BoNT/C-HCR and the neutrally charged lipids, phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylcholine (PC).

To further analyze the relative binding affinities of the BoNT/C-HCR domain with phospholipids, membrane arrays spotted with different concentrations of phosphoinositides were utilized. Consistent with the initial results, BoNT/C-HCR bound the negatively charged phosphoinositides on the membrane, but not the neutrally charged PI (Fig. 1B). BoNT/C-HCR showed a strong interaction with the monophosphoinositide species PI(3)P, PI(4)P, and PI(5)P and the diphosphoinositide species PI(3,5)P₂. Furthermore, BoNT/C-HCR binding with PI(5)P, and PI(3,5)P₂ was observed at low lipid concentrations (Fig. 1B), indicating that BoNT/C-HCR binds more tightly with PI(5)P and PI(3,5)P₂ than with PI(3)P, PI(4)P and PI(5)P on a membrane surface.

Liposomes more closely approximate the cell membrane structure and environment than membrane based assays, therefore the analysis of membrane interactions utilizing liposomes may better reflect *in vivo* molecular interactions [17]. Therefore, to confirm the interactions of BoNT/C-HCR with phosphoinositides, a liposome-binding assay was utilized. BoNT/C-HCR was incubated with liposomes composed of defined lipids and subjected to centrifugation. Proteins that bind to lipids will be spun down together with liposomes and found in the pellet. Distribution of bound and unbound protein was analyzed by SDS-PAGE. It was found that BoNT/C-HCR strongly associated with liposomes composed of phosphoinositides as indicated by the detection of greater than 70% of BoNT/C-HCR in the pellet fraction of liposomes composed of 100% natural phosphoinositides (Folch fraction I, Fig. 2A). In contrast, BoNT/CHCR was not found in the pellet when liposomes were omitted from the mixture.

Since phosphoinositides are usually present at low concentrations in membranes, interactions between BoNT/C-HCR and liposomes containing more physiologically relevant levels of phosphoinositides were tested. PC was used as a scaffold lipid for mixing with test phosphoinositides not only because it is the most common membrane phospholipid but also because of its low background binding with BoNT/C-HCR (Fig. 2A). We first tested whether BoNT/C-HCR interacts with the common phospholipids, phosphatidic acid (PA) and phosphatidylserine (PS). Using liposomes composed of 80 mol% PC and 20 mol% PA or PS, it was found that less than 25% of the BoNT/C-HCR associated with the liposomes (Fig. 2A), indicating that BoNT/C-HCR interacts only weakly with PA or PS. In contrast, when liposomes were made containing the individual phosphoinositides at a 20 mol% in a PC background, over 80% of the BoNT/C-HCR was recovered in the pellet fraction from PI(3)P, PI(4)P, PI(3,4)P₂, PI(4,5)P₂, or PI(3,5)P₂ enriched liposomes (Fig. 2B), indicating that BoNT/C-HCR has a strong binding affinity for these phosphoinositides. BoNT/C-HCR also associated, although to a lesser extent, with liposomes enriched with 20 mol% PI(5)P or PI(3,4,5)P₃ (Fig. 2B). When the concentration of individual phosphoinositides was further reduced to 5% of the total lipid in the liposomes (95 mol% PC), a strong association between BoNT/C-HCR and PI(3)P, PI(4)P enriched liposomes continued to be detected with almost no reduction in the recovery of BoNT/C-HCR in the pellet (Fig. 2C). The association of BoNT/C-HCR with PI(5)P, PI(3,4)P₂, PI(4,5)P₂, PI(3,5)P₂, and PI(3,4,5)P₃ was slightly reduced with 55–75% of the BoNT/C-HCR recovered in the pellet (Fig. 2C).

The results from the membrane dot-blot assay and the liposome-binding assay both demonstrate strong BoNT/C-HCR binding with phosphoinositides, however different phosphoinositide interactions are seen between the assays. The dot-blot assay detects a strong BoNT/C-HCR association with PI(5)P and PI(3,5)P₂. Whereas the liposome-binding assay shows the strongest BoNT/C-HCR association with the monophosphoinositides, PI(3)P and PI(4)P. These differences could result from the different state of the lipids in the two assays. Lipids are randomly immobilized on the membrane for dot-blot assays and no ordered structure can be assumed. However, in liposome-binding assays, both the structure and composition of liposomes more closely reflect the *in vivo* membrane environment with which the toxin interacts [17]. Regardless of which assay is used, both assays demonstrate a strong association of BoNT/C-HCR with phosphoinositides, but weakly or none with other lipids.

Protein and phosphoinositides interactions are commonly observed in cellular signaling transduction for membrane traffic and recognition [18]. For BoNT, interacting with additional lipids other than gangliosides may help to provide tighter membrane binding and better positioning of the translocation domain for subsequent membrane penetration, as suggested by Muraro *et al.* [13]. Protein binding promiscuously to negatively charged phosphoinositides is not uncommon for membrane-interacting proteins [18] and may provide an advantage for enhancing membrane recognition.

The exact region(s) that BoNT/C-HCR binds phosphoinositides is unclear. Muraro, *et al.* suggested that the Hcn subdomain of BoNT/A-HCR, specifically the S876-N929 region may be involved in the interactions [13]. From the crystal structure of BoNT/C-HCR [19], a similar region was revealed in residues S871-N926 and seems to have the potential to participate the binding. Co-crystal structure of BoNT/C-HCR with phosphoinositides would help to further confirm these hypotheses.

Highlights

- Binding interactions between BoNT/C-HCR and phospholipids was evaluated *in vitro*.

- ▶ BoNT/C-HCR specifically bound negatively charged phospholipids, preferentially phosphoinositides.
- ▶ Interactions between BoNT/C-HCR and phosphoinositides may facilitate tighter binding between neuronal membranes and BoNT/C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

BoNT	botulinum neurotoxin
BoNT/C-HCR	the receptor binding domain of BoNT serotype C
BoNT/D-HCR	the receptor binding domain of BoNT serotype D
Hc	BoNT C-terminal 100 kDa heavy chain
Hcc	the C-terminal sub-domain of HCR
Hcn	the N-terminal sub-domain of HCR
HCR	the receptor binding domain
Lc	BoNT N-terminal 50 kDa light chain
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PI(3)P	phosphatidylinositol 3-phosphate
PI(4)P	phosphatidylinositol 4-phosphate
PI(5)P	phosphatidylinositol 5-phosphate
PI(3,4)P2	phosphatidylinositol (3,4)-bisphosphate
PI(3,5)P2	phosphatidylinositol (3,5)-bisphosphate
PI(4,5)P2	Phosphatidylinositol (4,5)-bisphosphate
PI(3,4,5)P3	phosphatidylinositol (3,4,5)-trisphosphate
PS	phosphatidylserine
S1P	sphingosine 1-phosphate

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References

1. Lamanna C. The most poisonous poison. *Science*. 1959; 130:763–772. [PubMed: 14413547]
2. Schiavo G, Benfenati F, Poulain B, Rossetto O, Polverino de Laureto P, DasGupta BR, Montecucco C. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature*. 1992; 359:832–835. [PubMed: 1331807]
3. Aron SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Hauer J, Layton M, Lillibridge S, Osterholm MT, O'Toole T, Parker G, Perl TM, Russell PK, Swerdlow DL, Tonat K. Botulinum toxin as a biological weapon: medical and public health management. *JAMA*. 2001; 285:1059–1070. [PubMed: 11209178]
4. Tonello F, Morante S, Rossetto O, Schiavo G, Montecucco C. Tetanus and botulism neurotoxins: a novel group of zinc-endopeptidases. *Adv Exp Med Biol*. 1996; 389:251–260. [PubMed: 8861019]
5. Montecucco C. How Do Tetanus and Botulinum Toxins Bind to Neuronal Membranes. *Trends Biochem Sci*. 1986; 11:314–317.
6. Stenmark P, Dupuy J, Imamura A, Kiso M, Stevens RC. Crystal structure of botulinum neurotoxin type A in complex with the cell surface co-receptor GT1b-insight into the toxin-neuron interaction. *PLoS Pathog*. 2008; 4:e1000129. [PubMed: 18704164]
7. Strotmeier J, Lee K, Volker AK, Mahrhold S, Zong Y, Zeiser J, Zhou J, Pich A, Bigalke H, Binz T, Rummel A, Jin R. Botulinum neurotoxin serotype D attacks neurons via two carbohydrate-binding sites in a ganglioside-dependent manner. *Biochem J*. 2010; 431:207–216. [PubMed: 20704566]
8. Strotmeier J, Gu S, Jutzi S, Mahrhold S, Zhou J, Pich A, Eichner T, Bigalke H, Rummel A, Jin R, Binz T. The biological activity of botulinum neurotoxin type C is dependent upon novel types of ganglioside binding sites. *Mol Microbiol*. 2011; 81:143–156. [PubMed: 21542861]
9. Benson MA, Fu Z, Kim JJ, Baldwin MR. Unique ganglioside recognition strategies for clostridial neurotoxins. *J Biol Chem*. 2011
10. Peng L, Tepp WH, Johnson EA, Dong M. Botulinum Neurotoxin D Uses Synaptic Vesicle Protein SV2 and Gangliosides as Receptors. *PLoS Pathog*. 2011; 7:e1002008. [PubMed: 21483489]
11. Montal M. Botulinum neurotoxin: a marvel of protein design. *Annu Rev Biochem*. 2010; 79:591–617. [PubMed: 20233039]
12. Tsukamoto K, Kohda T, Mukamoto M, Takeuchi K, Ihara H, Saito M, Kozaki S. Binding of Clostridium botulinum type C and D neurotoxins to ganglioside and phospholipid. Novel insights into the receptor for clostridial neurotoxins. *J Biol Chem*. 2005; 280:35164–35171. [PubMed: 16115873]
13. Muraro L, Tosatto S, Motterlini L, Rossetto O, Montecucco C. The N-terminal half of the receptor domain of botulinum neurotoxin A binds to microdomains of the plasma membrane. *Biochem Biophys Res Commun*. 2009; 380:76–80. [PubMed: 19161982]
14. Zhang Y, Gao X, Qin L, Buchko GW, Robinson H, Varnum SM. High-level expression, purification, crystallization and preliminary X-ray crystallographic studies of the receptor-binding domain of botulinum neurotoxin serotype D. *Acta Crystallogr Sect F Struct Biol Cryst Commun*. 2010; 66:1610–1613.
15. Zhang Y, Buchko GW, Qin L, Robinson H, Varnum SM. Structural analysis of the receptor binding domain of botulinum neurotoxin serotype D. *Biochem Biophys Res Commun*. 2010; 401:498–503. [PubMed: 20858456]
16. Zhang Y, Buchko GW, Qin L, Robinson H, Varnum SM. Crystal structure of the receptor binding domain of the botulinum C-D mosaic neurotoxin reveals potential roles of lysines 1118 and 1136 in membrane interactions. *Biochem Biophys Res Commun*. 2011; 404:407–412. [PubMed: 21130733]
17. DiNitto JP, Cronin TC, Lambright DG. Membrane recognition and targeting by lipid-binding domains. *Sci STKE*. 2003; 2003:re16. [PubMed: 14679290]
18. Lemmon MA. Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol*. 2008; 9:99–111. [PubMed: 18216767]
19. Karalewitz AP, Kroken AR, Fu Z, Baldwin MR, Kim JJ, Barbieri JT. Identification of a unique ganglioside binding loop within botulinum neurotoxins C and D-SA. *Biochemistry*. 2010; 49:8117–8126. [PubMed: 20731382]

Fig. 1. BoNT/C-HCR binds preferentially with phosphoinositides. BoNT/C-HCR recombinant protein was overlaid onto nitrocellulose membranes and proteins bound to lipids were detected with the anti-His tag antibody. **(A)** PIP strip pre-spotted with 15 different kinds of lipid molecules (100 pmol each). **(B)** PIP array pre-spotted with different concentrations of lipids.

Fig. 2.

Binding of BoNT/C-HCR to phosphoinositide-containing liposomes. BoNT/C-HCR recombinant protein was incubated with liposomes containing various amounts of phospholipids and subjected to centrifugation to spin down the liposomes. Pellets were redissolved in TBS buffer to the same volume as the supernatant. Equal volumes of the pellet and supernatant fractions were loaded and resolved by SDS-PAGE, shown in upper panels. Note that protein bound liposomes is in the pellet (P) fraction and non-bound protein is in the supernatant fraction (S). Lower panels are graphic representations depicting the percentage of BoNT/C-HCR binding to liposomes containing: **(A)** Folch I: brain lipids extract containing a mixture of diphosphoinositide, triphosphoinositide, phosphatidylinositol and phosphatidylserine. PC: 100% PC; PA: 20% PA plus 80% PC; PS: 20% PS plus 80% PC. **(B)** 20% indicated phosphoinositides (plus 80% PC); **(C)** 5% indicated phosphoinositides (plus 95% PC). All data represent results from three independent experiments.