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Lipid and cationic polymer based transduction of botulinum holotoxin, or toxin protease alone, extends the target cell range and improves the efficiency of intoxication

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ABSTRACT

Botulinum neurotoxin (BoNT) heavy chain (Hc) facilitates receptor-mediated endocytosis into neuronal cells and transport of the light chain (Lc) protease to the cytosol where neurotransmission is inhibited as a result of SNARE protein cleavage. Here we show that the role of BoNT Hc in cell intoxication can be replaced by commercial lipid-based and polycationic polymer DNA transfection reagents. BoNT "transduction" by these reagents permits efficient intoxication of neuronal cells as well as some non-neuronal cell lines normally refractory to BoNT. Surprisingly, the reagents facilitate delivery of recombinant BoNT Lc protease to the cytosol of both neuronal and non-neuronal cells in the absence of BoNT Hc, and with sensitivities approaching that of BoNT holotoxin. Transduction of BoNT, as with natural intoxication, is inhibited by bafilomycin A1, methylamine and ammonium chloride indicating that both pathways require endosome acidification. DNA transfection reagents facilitate intoxication by holotoxins, or isolated Lc proteases, of all three BoNT serotypes tested (A, B, E). These results suggest that lipid and cationic polymer transfection reagents facilitate cytosolic delivery of BoNT holotoxins and isolated Lc proteases by an endosomal uptake pathway.

1. Introduction

Botulinum neurotoxins (BoNTs) are extremely potent protein toxins and cause flaccid paralysis by entering motor neurons and inhibiting vesicular neurotransmitter release. There are seven BoNT serotypes, A–G, each cleaving soluble NSF attachment receptor (SNARE) proteins SNAP25, synaptobrevin, and/or syntaxin at different sites to inhibit neurotransmitter exocytosis (Binz et al., 1994; Blasi et al., 1993; Schiavo et al., 1992, 1993a,b). Each BoNT contains a heavy chain (Hc) and light chain (Lc) tethered by a single disulfide bond (DasGupta and Sugiyama, 1972). The Hc includes a receptor binding domain and a translocation domain while the Lc contains zinc metalloprotease activity (Hoch et al., 1985; Lacy et al., 1998; Montecucco and Schiavo, 1993). During the intoxication process, botulinum toxin binds with high specificity to receptors on the nerve termini at the neuromuscular junction (Kozaki et al., 1989; Lacy et al., 1998; Lalli et al., 1999). These receptors for BoNT include both a ganglioside and a cell surface protein (Kitamura et al., 1980; Montecucco, 1986; Nishiki et al., 1996). The synaptic vesicle protein 2 (SV2) (Dong et al., 2008; Mahrhold et al., 2006) is the receptor for BoNT/A while synaptotagmins I and II are the protein receptors for serotypes B and G, respectively (Dong et al., 2007; Nishiki et al., 1994, 1996; Rummel et al., 2004). Following receptor-mediated endocytosis (Binz and Rummel, 2009; Humeau et al., 2000; Simpson, 1980, 1981, 2000; Verderio et al., 2007) and endosome formation (Fisher and Montal, 2006; Keller et al., 2004; Montecucco et al., 1994), the Lc is

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translocated across vesicle membrane into the cytosol through a protein channel formed by the amino terminus of the Hc (Blaustein et al., 1987; Blocker et al., 2003; Cai et al., 2006b; Fisher and Montal, 2006; Hoch et al., 1985; Humeau et al., 2000; Koriazova and Montal, 2003; Schmid et al., 1993; Shone et al., 1987; Simpson, 1980, 1981). The translocation of the Lc to the cytosol occurs following a conformational change that is induced by acidification of the endosome (Cai et al., 2006b; Hoch et al., 1985). Drugs that inhibit endosome acidification, such as bafilomycin A1, are antagonists of BoNT intoxication (Deshpande et al., 1997; Simpson, 1983; Simpson et al., 1994). Once in the cytosol, the different BoNT Lcs cleave and inactivate their SNARE protein substrates leading to intoxication.

Botulinum toxin proteases are useful reagents in studies on cellular transport and secretion because of their ability to specifically cleave SNARE proteins. Because of the potential of BoNT as a weapon in bioterrorism, major research efforts are also underway to develop both antitoxins and antidotes for botulism. Botulinum toxin research, though, has been hampered by the fact that the toxins naturally enter only those cells which express the surface receptors recognized by the BoNT serotype under study. These receptors are generally present in significant amounts on primary neuronal cells but are often poorly expressed or even lacking in neuronal cell lines. Neuroblastoma cell lines are available that can become intoxicated but often require high doses of toxin to produce evidence of intoxication if it occurs at all (Ibanez et al., 2004; Purkiss et al., 2001). Primary neuronal cells are sensitive to most BoNT serotypes although these are difficult and costly to prepare and to maintain (Keller et al., 2004).

To overcome the restrictions to BoNT intoxication that exist because of the lack or low level of appropriate receptors on target cell lines, researchers have employed alternative methods to deliver BoNT proteases to the cytosol. One method has been to introduce plasmids which drive expression of BoNT proteases within transfected cells (Fernandez-Salas et al., 2004; He et al., 2008; Huang et al., 1998, 2001; Ji et al., 2002; Land et al., 1997; Shu et al., 2008). Electroporation of BoNT holotoxin or isolated Lc proteases has been employed for functional delivery of the Lc protease to the cytosol of secretory cells (Boyd et al., 1995; Erdal et al., 1995; Wang and Nadler, 2007) and streptolysin permeabilization has been used for delivery of BoNT holotoxins or Lc proteases to cells and synaptosomes (Gonelle-Gispert et al., 1999; Leung et al., 1998; Pickett et al., 2007; Sadoul et al., 1997) in situations where they are tested immediately after treatment. In these methods, the amount of Lc protease delivered to the cytosol is not easily controlled and likely much higher than occurs during natural intoxication. None of these methods deliver toxin to the cytosol through an endosomal uptake process as occurs in natural intoxication. Clearly a method that promotes delivery of BoNT Lc protease to the cytosol of most neuronal and non-neuronal cells through endosome-mediated uptake would offer a valuable new tool for research employing BoNTs.

In studies of botulinum intoxication in cultured neuronal cell, we observed much more efficient BoNT/A intoxication occurred when the cells had been recently subjected to DNA transfection by lipofection. These reagents are routinely used for the delivery of DNA into cells by forming DNA/lipofection complexes that enter cells via the endocytic pathway (Zabner et al., 1995; Zhou and Huang, 1994). Transfection is typically independent of endosomal acidification and involves interactions between cationic lipid in the liposome and anionic lipid in the endosome (Zelphati and Szoka, 1996). Physical dissociation of DNA from the DNA–lipid complex and release of DNA from the endosome into the cytosol is a critical step in DNA transfection (Zelphati and Szoka, 1996). In this study, we tested the ability of standard DNA transfection reagents to deliver both BoNT holotoxins and recombinant BoNT Lc proteases to neuronal and non-neuronal cells. These reagents enabled potent, endosome-mediated, BoNT intoxication of most cell lines tested without compromising cell viability and are independent of the need for BoNT receptors. This technology opens new avenues for investigating BoNT holotoxin function in a wide variety of cell lines as well as performing cell-based drug assays without select agent requirements.

2. Materials and methods

2.1. Cell culture and reagents

M17 (ATCC# CRL-2267) cells were maintained in DMEM (Gibco) containing 10% fetal bovine serum (FBS) (Gibco). MEME (Gibco) plus 10% FBS was used for culturing Neuro2a (ATCC# CCL-131) (abbreviated N2A) and HEK293 (ATCC# CRL-1573) cells. HIT-T15 (ATCC# CRL-1777) cells were cultured in F12K (Gibco) containing 10% horse serum and 5% FBS. A total of 6×10^4 cells were seeded onto each well of a 24-well plate and maintained at 37 °C. After 72 h, culture medium was replaced with fresh medium before experimental treatments.

Primary cultures of cerebellar granule cells were prepared from 7 day-old Sprague–Dawley rats (Welch et al., 2000) essentially by the methods of Farkas et al. (2004). Briefly, after aseptically removing cerebella from the skulls, tissue was freed from meninges and incubated in 0.05% trypsin solution for 10 min at RT. After a brief centrifugation, cells were triturated in DMEM/F12 containing 10% FBS and filtered through a sterile cell strainer mesh with 40 μm pore size (BD Falcon) (Foran et al., 2003; Kornyei et al., 1998; Sabbieti et al., 2004). Cell number was determined by trypan blue exclusion, and cells were seeded onto a poly-L-lysine (PLL) 1 g/cm^2 laminin (Sigma) coated six-well plate with DMEM containing 10% FBS, 25 mM KCl, 2 mM Glutamax, and 100 g/ml gentamicin (Gibco). The cultures were maintained at 37 °C in a humidified atmosphere of 6% CO_2. After 24 h of culturing, cytosine arabinoside (Sigma) was added to a final concentration of 20 μM to prevent astrocytic proliferation. The neurons were cultured for 7–8 days before use.

FuGene-HD (Roche), Lipofectamine 2000 (Invitrogen) and PEI average molecular weights 0.6, 1.8, 10, 70 kDa (Alfa Aesar) and 25 kDa (Sigma) were used for transfection and transduction as recommended by the manufacturer except where indicated. Bafilomycin A1 was obtained from Tocris Cookson Inc. Methylamine hydrochloride and ammonium chloride were obtained from Sigma.