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Low pH-Induced Pore Formation by the T Domain of Botulinum Toxin Type A is Dependent upon NaCl Concentration

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Abstract Botulinum neurotoxins (BoNTs) undergo low pH-triggered membrane insertion, resulting in the translocation of their light (catalytic) chains into the cytoplasm. The T (translocation) domain of the BoNT heavy chain is believed to carry out translocation. Here, the behavior of isolated T domain from BoNT type A has been characterized, both in solution and when associated with model membranes. When BoNT T domain prepared in the detergent dodecylmaltoside was diluted into aqueous solution, it exhibited a low pH-dependent conformational change below pH 6. At low pH the T domain associated with, and formed pores within, model membrane vesicles composed of 30 mol% dioleoylphosphatidylglycerol/70 mol% dioleoylphosphatidylcholine. Although T domain interacted with vesicles at low (50 mM) and high (400 mM) NaCl concentrations, the interaction required much less lipid at low salt. However, even at high lipid concentrations pore formation was much more pronounced at low NaCl concentrations than at high NaCl concentration. Increasing salt concentration after insertion in the presence of 50 mM NaCl did not decrease pore formation. A similar effect of NaCl concentration upon pore formation was observed in vesicles composed solely of dioleoylphosphatidylcholine, showing that the effect of NaCl did not solely involve modulation of electrostatic interactions between protein and anionic lipids. These results indicate that some feature of membrane-bound T domain tertiary structure critical for pore formation is highly dependent upon salt concentration.

Keywords Lipid protein interaction · Membrane biophysics · Membrane protein · Protein toxin

Introduction

Clostridium botulinum secretes botulinum neurotoxin (BoNT) serotypes A–G. BoNTs are produced as single inactive polypeptide chains of ~150 kDa and cleaved by proteases into a heavy chain (HC) of ~100 kDa linked by a disulfide bond to a light chain (LC) of ~50 kDa. The high-resolution structure of BoNTs has been solved (Kumaran et al. 2009; Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000). The C-terminal half of the HC is the receptor binding portion of the BoNT protein and is composed of two subdomains, an N terminal lectin-like jelly-roll domain and a C-terminal β-trefoil domain. The carbohydrate portion of glycolipids (Swaminathan and Eswaramoorthy 2000; Yowler et al. 2002; Yowler and Schengrund 2004) and the extracellular portion of synaptic vesicle membrane proteins bind to the β-trefoil domain (Baldwin and Barbieri 2007; Hanson and Stevens 2000). The N-terminal half of the HC is the translocation (T) domain, believed to be responsible for translocation of the LC across membranes. It is composed of many alpha-helices plus an extended N-terminal belt that

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surrounds the LC (Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000). Upon receptor-mediated endocytosis, exposure of the BoNT protein to the low pH in an endocytic compartment is believed to trigger translocation (Lalli et al. 2003). LC proteins are proteases, and once they reach the cytoplasm, they attack either synaptosome-associated 25-kDa protein (SNAP-25; BoNT types A, C and E) or synaptobrevin, also called vesicle-associated membrane protein (VAMP; BoNT types B, D, F and G) (Schiavo et al. 2000).

Low pH-triggered membrane insertion of a translocation domain is a key step in the translocation of protein toxin catalytic domains in several cases (Koehler and Collier 1991; Lalli et al. 2003; London 1992). In the case of diphtheria toxin, hydrophobic helices within the T domain are believed to insert and form the translocation pathway through which the catalytic chain must pass (Choe et al. 1992). In the case of anthrax toxin, membrane-inserted protective antigen forms a large beta-barrel with a pore which is the likely pathway for translocation of the catalytic subunit (which can be either lethal factor or edema factor) (Krantz et al. 2006; Nassi et al. 2002). However, in the case of the BoNT T domain, the exact sequences involved in forming the translocation pathway and their structural form are unknown. Although it has a helical structure, like the T domain of diphtheria toxin, the BoNT T domain differs from that of diphtheria toxin in that it has only two hydrophobic sequences and they are not packaged into hydrophobic helices. Furthermore, the BoNT T domain is much larger than that of diphtheria toxin and has to translocate a much larger catalytic subunit. This suggests that there must be some important differences in their functioning. On the other hand, there may also be mechanistic similarities as a chaperone-like function has been proposed for both diphtheria toxin and BoNT T domains (Brunger et al. 2007; Fischer and Montal 2007; Hammond et al. 2002; Koriazova and Montal 2003; Ren et al. 1999).

Prior studies have shown that BoNT undergoes membrane insertion and forms pores at low pH (Fu et al. 2002; Koriazova and Montal 2003; Montecucco et al. 1989; Puhar et al. 2004; Schiavo et al. 1990). However, it would be advantageous to carry out such studies with isolated BoNT T domain. Comparison of the properties of isolated T domain to those of T domain attached to other domains would allow determination of what properties are intrinsic to the T domain and how domain–domain interactions affect T domain conformation and translocation. Furthermore, membrane-inserted isolated T domain would be an ideal subject for studies defining the topography of membrane-inserted T domain and, thus, the likely structure of the translocation pathway. Finally, isolated T domain should also be a suitable system for helping to identify compounds that interfere with the translocation process.

However, relatively little is known about the behavior of isolated BoNT T domain. One early study confirming that a T domain fragment isolated from whole BoNT by proteolysis retains pore-forming properties (Blaustein et al. 1987). A recent study by Galloux et al. (2008) showed that the isolated T domain has the ability to insert into model membrane vesicles at low pH. Here, we report on the further characterization of properties of the membrane-inserted isolated T domain from botulinum toxin type A. We confirm that the isolated BoNT T domain maintains pH-dependent membrane insertion and pore-formation properties similar to those found in the intact protein. Furthermore, the properties of the BoNT T domain show an interesting dependence upon NaCl concentration that may be an important clue to the control of BoNT T domain conformation in membranes and the structure of the BoNT T domain pore.

Experimental Procedures

Materials

Dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylglycerol (DOPG) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(1-pyrenesulfonyl) (pyrene-DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Lipid concentrations were determined by dry weight. *N*-[(4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indecene-3-yl) methyl]iodoacetamide (BODIPY-FL C1-IA, BODIPY-IA) labeled streptavidin (BODIPY-SA) was purchased (custom order) from Molecular Probes (Eugene, OR). Biocytin (ε-biotinyl-lysine) was from Sigma-Aldrich (St. Louis, MO). All of the other chemicals were reagent-grade.

Preparation of Isolated Botulinum Toxin Type A T Domain

"Wild-type" BoNT T (residues 448–870) and BoNT T domain containing two mutations (C453A, C790A) to remove the two cysteines were used. In both cases, the protein carried a 19-residue 6X-His tag on the N terminus.

Cloning of Translocation Domain

A pET15b vector, harboring gene for the full-length HC (BAHc-pET15b) kindly provided by Dr. Bal Ram Singh (University of Massachusetts, Dartmouth), was used as a template. The forward primer had an *Nhe*I restriction site at the 5′ position and the reverse, a *Bam*HI site (forward [F] 5′-GGCCGGATCCCCGCTAGCGCATTAAATGATTTATG-3′ and reverse [R] 5′-TATAGGATCCTTACTTAATA

TATTC-3′). PCR-amplified fragments were cloned first to PCR 2.1 vector using a TA cloning kit (Invitrogen, Carlsbad, CA). The DNA fragment of interest was digested out using the same enzymes and ligated to pET28b vector within the same restriction sites. Clones were confirmed by double digestion (*Nhe*I and *Bam*HI), followed by the Big Dye terminator cycle DNA sequencing (Applied Biosystems, Foster City, CA). The clone possessed an N-terminal 6X-His tag coding sequence. A correct clone was transformed in BL21 (DE3) RIPL cells for protein expression.

Cysteine Mutagenesis

The mutant primers for C453A had the sequences F 5′-GGC ATT AAA TGA TTT A **GCT** AT CAA AGT TAA TAA TTG GGA C-3′ and R 5′-GT CCC AAT TAT TAA CTT TGA T **AGC** TA AAT CAT TTA ATG CC-3′ and for C790A they had the sequences F 5′-TGA ATC AA**G CC**T CTG TTT CAT ATT TAA TG-3′ and R 5′-CA TTA AAT ATG AAA CAG A**GG C**TT GAT TCA-3′; these were used with a site-directed mutagenesis kit (Stratagene, La Jolla, CA) to generate mutations. The mutants were confirmed for the correct sequence by DNA sequencing and transformed to BL21 (DE3) RIPL cells for expression.

Expression and Purification of T Domain and Mutants

The native and mutant proteins were expressed in autoinduction media (Studier 2005). Cultures were grown at 37°C until OD_{600} reached 0.6 and then at 20°C for 14–16 h. The cell pellet was lysed in 30 ml Tris buffer (50 mM, 200 mM NaCl, pH 7.5) containing 3 ml Bugbuster (Novagen, Gibbstown, NJ), 2 protease inhibitor cocktail tablet (Roche, Indianapolis, IN), 10 µl benzonase (Novagen) and 2 mM β-mercaptoethanol at room temperature for 30 min. The resulting suspension was centrifuged at 16,000 rpm for 30 min to remove soluble proteins. The T domain protein is mostly found in an inclusion body pellet. The pellets were solubilized in Tris buffer containing 1% (w/v) dodecylmaltoside (DDM) detergent at room temperature and then centrifuged at 16,000 rpm. The T domain was purified from the supernatant by chromatography on Ni–NTA agarose using an imidazole gradient in Tris buffer containing 0.1% DDM. Fractions containing the maximum amount of proteins were pooled, applied to a Sepharose fast-flow anion exchange column (void volume 20 ml) and eluted using an NaCl gradient in Tris buffer (25 mM [pH 7.5], containing 0.1% DDM detergent). The T domain eluted as a sharp pure peak. The pure protein fractions were then pooled and concentrated in 100-kDa cutoff centricon filters (Millipore, Billarica, MA). The protein was further purified by size-exclusion chromatography in superdex200 16/60 column (GE Healthcare, Piscataway, NJ) in Tris buffer 2 (25 mM, 100 mM NaCl [pH 7.5]). Fractions containing pure protein were pooled, concentrated and dialyzed in 100-kDa cutoff Dispo-dialyser bags (Millipore) for 24 h. Over 10 mg of pure T domain was obtained from the pellet from a 1-l culture. The final stock protein solution contained 2–4 mg/ml BoNT T domain in 20 mM Tris–Cl (pH 7.8), 200 mM NaCl and 0.1 w/v% DDM.

Fluorescence Measurements

BoNT fluorescence was measured on a Spex tau-2 Fluorolog spectrofluorimeter (Horiba Jobin–Yvon, Edison, NJ) operating in steady-state mode. Fluorescence measurements were performed at room temperature using excitation and emission slit widths of 2.5 mm (bandpass 4.5 nm) and 5 mm (bandpass 9 nm), respectively. For most fluorescence measurements, cuvettes with an excitation path length of 10 mm and an emission path length of 4 mm were used. For pH and lipid vesicle titration experiments, cuvettes with an excitation path length of 10 mm and an emission path length of 10 mm were used. Trp fluorescence was excited at 280 or 295 nm.

Circular Dichroism Measurements

Circular dichroism (CD) spectra were measured on a Jasco (Tokyo, Japan) J-715 spectropolarimeter. Quartz cuvettes with a path length of 1 mm were used. To calculate mean residue molecular ellipticity, a molecular weight of 48.8 kDa and 425 residues were used. Each final spectrum was an average of 50 scans.

Preparation of Lipid Vesicles

Large unilamellar vesicles (LUVs) were prepared as follows: 3.5 µmol DOPC and 1.5 µmol DOPG (or 1.25 µmol DOPG and 0.25 µmol pyrene-DOPE used in place of 1.5 µmol DOPG) dissolved in chloroform were dried at about 60°C while flushing with nitrogen gas. Samples were further dried under high vacuum for 1 h. Then, 500 µl 10 mM Tris–Cl (pH 7.8), 150 mM NaCl (Tris buffer) and 10 mg of the detergent *n*-octyl-β-D-glucoside were added to the lipid mixture. The lipid was then dissolved in the detergent with vortexing. The resulting solution was dialyzed overnight against 4 l Tris buffer, with no buffer change, using a molecular weight cutoff 8000 Spectra/Por dialysis membrane from Spectrum Laboratories (Greensboro, NC). Lipid concentrations given below assume that the change in sample volume during dialysis was negligible. The protocol for forming vesicles composed solely of DOPC was almost identical, except that sample formation started with 10 µmol DOPC, which was later hydrated with 1 ml of Tris buffer.

LUVs with trapped BODIPY-SA were prepared by an analogous procedure except that the dialyzed preparation was chromatographed on Sepharose 4BCL to remove residual external BODIPY-SA (Lai et al. 2008; Zhao and London 2005). (In this case the fluorescence of a trace amount of rhodamine-labeled lipid was used to determine final lipid concentration.) This procedure produced intact vesicles as judged by the inability of biocytin to react with BODIPY-labeled SA entrapped within the vesicles in the absence of BoNT T domain (see below).

Trp Fluorescence Spectra of BoNT T

For Trp fluorescence spectra, samples of 800 µl were prepared by adding an aliquot of BoNT (to give a final concentration 20 µg/ml) to a solution of 170 mM Na acetate (NaAc, pH 4.0) containing either 50 or 400 mM NaCl or to a solution of these buffers mixed with an aliquot of LUV containing 200 µM 30 mol% DOPG/70 mol% DOPC. After incubating for 20 min, spectra were measured by exciting Trp at 295 nm and measuring emission intensity over the range 310–400 nm.

pH Dependence of BoNT T Domain Fluorescence

Samples of 2 ml containing 20 µg/ml BoNT T added to a buffer of 20 mM Tris–Cl (pH 8.0), plus 50 or 400 mM NaCl, were prepared with or without LUV containing 200 µM 30 mol% DOPG/70 mol% DOPC as described above. The samples were titrated with small aliquots of either pure acetic acid (HAc) or 10% (v/v) HAc in water. Fluorescence was measured about 4 min after each aliquot was added. (A control experiment indicated this was long enough for fluorescence to stabilize [not shown].) The pH of the samples was estimated by measuring the pH (using a pH meter) of a parallel solution without protein but otherwise titrated in an identical manner. The samples were excited at 295 nm, and fluorescence intensity was measured at 340 nm after each aliquot of acid was added. In addition, shifts in emission spectra were detected by dividing fluorescence intensity at 330 nm by fluorescence intensity at 350 nm. Duplicate experiments were carried out.

Lipid Titration

For lipid vesicle titration, small aliquots from a sample of LUVs containing 10 mM lipid (either 30 mol% DOPG/70 mol% DOPC or 5 mol% pyrene-DOPC, 25 mol% DOPG/70 mol% DOPC) dispersed in 10 mM Tris–Cl (pH 7.8), 150 mM NaCl were titrated into a 2-ml sample containing 20 µg/ml BoNT T diluted into 170 mM NaAc (pH 4.0), 50 or 400 mM NaCl. After each aliquot was added, the sample was incubated for 4 min and then fluorescence was measured. Fluorescence was excited at 280 nm and the emission was collected at 340 nm.

BoNT T Pore Formation

Pore formation by BoNT T was detected using an assay similar to that used in previous studies of diphtheria toxin T domain pore formation (Lai et al. 2008; Zhao and London 2005). Briefly, the desired amount of BoNT T (diluted from the stock solution with storage buffer as to be contained in an 8-µl aliquot) was added to a sample containing ~66 ng/ml BODIPY-SA trapped within 100 µM 70% DOPC/30% DOPG LUV. This sample was prepared by mixing 25 µl of a stock preparation of LUVs containing trapped BODIPY-SA (stock solution contained 3.23 mM LUV and 2.12 µg/ml BODIPY-SA in 10 mM Tris–Cl [pH 7.8], 150 mM NaCl) with 767 µl of a solution containing the desired concentration of NaCl and one of the following buffers: 170 mM NaAc (pH 3.5–5.0), 170 mM MES (pH 5.5–6.5) or 170 mM Tris–Cl (pH 7.0–8.0). (Buffer pH was adjusted after NaCl addition.) After a 20-min incubation to allow the BoNT T domain to insert into the LUV, 20 nM biocytin was added to the exterior of the vesicles. The change in BODIPY fluorescence (excitation wavelength 488 nm, emission wavelength 516 nm) was then measured as a function of time. For the experiment in which NaCl concentration was changed, BoNT T was added to 743 µl of a sample containing the desired buffer, 50 mM NaCl and an aliquot of trapped BODIPY-SA and lipid, giving the same final concentrations as above. After a 20-min incubation, 57 µl 5 M NaCl buffer (5 M NaCl dissolved in the appropriate buffer) was added to give a final NaCl concentration of 400 mM. After an additional 10-min incubation, 20 nM biocytin (typically 4 µl from a 4 µM stock solution) was added and the change in BODIPY fluorescence measured. The protocol used for DOPC LUV was identical to that for the 70% DOPC/30% DOPG LUV, except that twice as much BoNT T domain was used and we did not quantify the amount of trapped BODIPY-SA in this case.

Results

Effect of pH, Lipid and NaCl upon BoNT T Domain

The Trp fluorescence of protein toxins has proven to be a sensitive method to study their conformation and membrane interaction (Blewitt et al. 1985; Malenbaum et al. 1998; Wang et al. 1997a), and we measured the native Trp fluorescence of the BoNT type A T domain (which has four Trp residues located in various positions along its sequence). Unless otherwise noted, Cys-less BoNT T domain in which the Cys residues at positions 453 and 790 were mutated to Ala was used.

Figure 1 shows the Trp emission spectra of Cys-less BoNT T domain near neutral and low pH and BoNT in the presence and absence of LUVs composed of 70 mol% DOPC/30 mol% DOPG. In solution at a low (50 mM) NaCl concentration, BoNT T domain fluorescence was relatively red-shifted, with a λ_{max} close to 340 nm. The

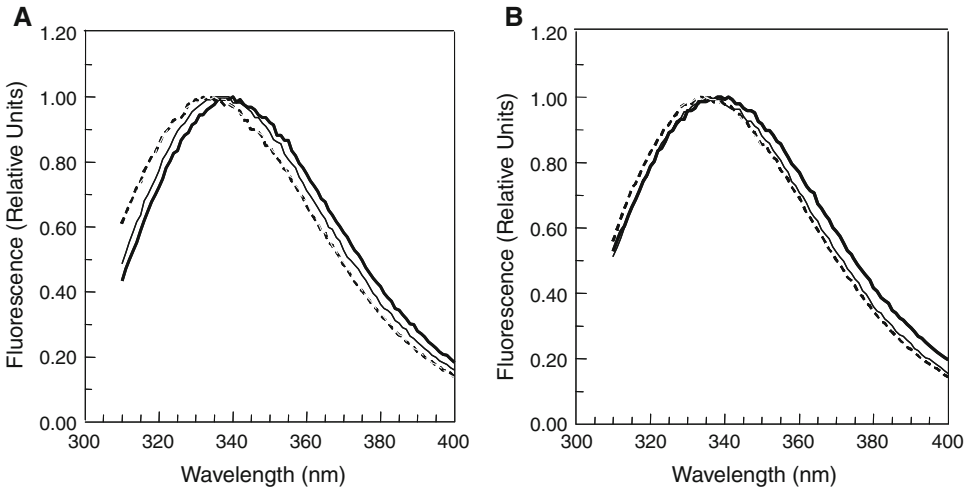

Fig. 1 BoNT T domain fluorescence spectra. **a** Fluorescence spectra in the presence of 50 mM NaCl. **b** Fluorescence spectra in the presence of 400 mM NaCl. Samples contained BoNT T domain in 70 mM Tris–Cl at pH 8.0 without lipid (*thick solid line*), 170 mM NaAc at pH 4.0 without lipid (*thin solid line*) and 170 mM NaAc pH 4.0 in the presence of LUV composed of 200 μM 70 mol% DOPC/30 mol% DOPG (*dashed line*). Spectra have been normalized so that the intensity at the emission λ_{max} is equal

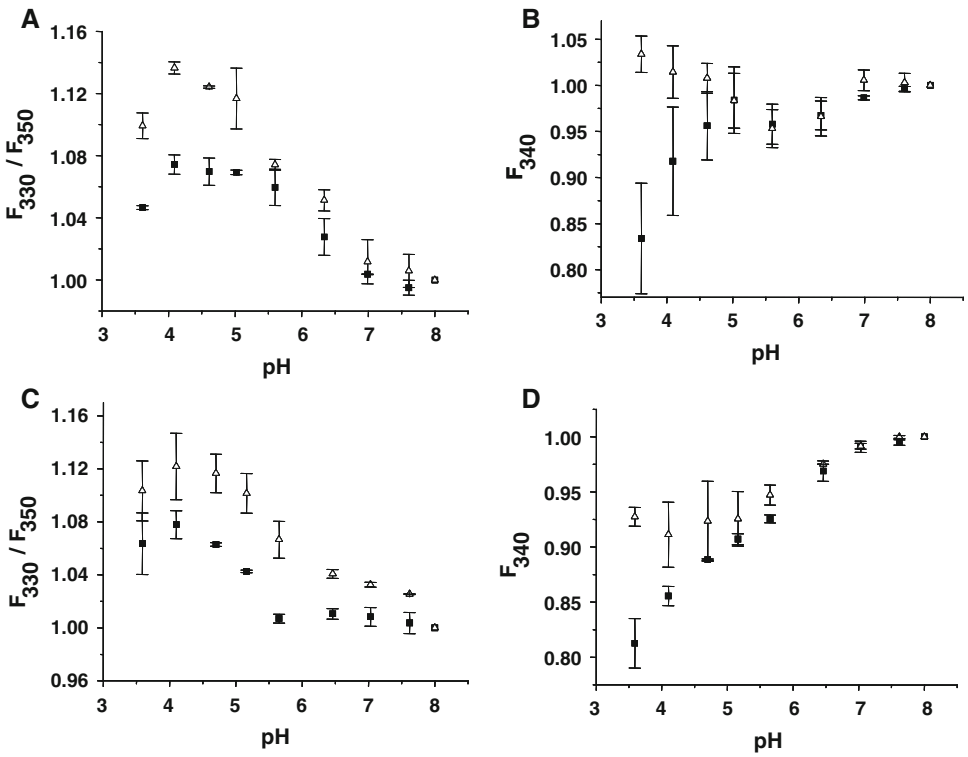

Fig. 2 Effect of pH upon BoNT T domain Trp fluorescence properties. **a** Ratio of fluorescence emission intensity at 330 nm to that at 350 nm in the presence of 50 mM NaCl. **b** Fluorescence intensity at 340 nm in the presence of 50 mM NaCl. **c** Ratio of fluorescence emission intensity at 330 nm to that at 350 nm in the presence of 400 mM NaCl. **d** Fluorescence intensity at 340 nm in the presence of 400 mM NaCl. Samples were prepared in 20 mM Tris–Cl, pH 8.0, in the presence (*open triangles*) or absence (*filled squares*) of LUVs composed of 200 μM 70% DOPC/30% DOPG LUV; and the pH was decreased with aliquots (0.5–5 μl) of acetic acid. Sample dilution by the end of the titration was negligible (<20 μl). Average values and range of duplicates are shown

presence of lipid vesicles did not shift fluorescence λ_{max} to a significant degree at neutral pH (not shown). In contrast, in solution at low pH, there was a blue shift in the fluorescence spectrum of about 2 nm. This suggests that there is a conformational change in which the average Trp environment became more hydrophobic. Figure 1 also shows that the blue shift at low pH was 2–3 nm greater in the presence of lipid, indicative of interaction of the protein with the LUV. Similar behaviors in solution and in the presence of lipid vesicles were observed for wild-type BoNT T domain (not shown). Behavior at a higher (400 mM) NaCl concentration (studied because of the effect of salt concentration upon pore formation, see below) was similar to that at 50 mM NaCl.

Figure 2 shows that when the pH dependence of the fluorescence changes was measured, a distinct transition in Trp fluorescence properties was observed at low pH. The blue shift in the emission maximum (measured by the ratio of emission at 330 nm to that at 350 nm, which is more sensitive than λ_{max}) exhibited a midpoint near pH 6 (at 400 mM NaCl) (Fig. 2a) or pH 5 (at 50 mM NaCl) (Fig. 2c) BoNT in the presence or absence of lipid. Again, the blue shift was greater in the presence of lipid. The intensity of Trp fluorescence (measured at a single fixed wavelength) decreased at low pH in solution. This decrease was largely suppressed in the presence of LUV.

The effect of the presence of LUV upon BoNT T domain fluorescence strongly suggests that the protein interacts with lipid vesicles at low pH. To measure the strength of this interaction, binding of BoNT T domain to vesicles was assessed by measuring the extent of fluorescence resonance energy transfer (FRET) from Trp to a pyrene-headgroup labeled lipid (pyrene-DOPE) incorporated into the vesicles. Figure 3 shows that when LUVs were titrated into a solution containing BoNT T domain there was a significant amount of FRET that tended to saturate at high lipid concentrations. Wild-type BoNT T domain exhibited similar behavior (Supplemental Fig. 1a). The level of FRET at near-saturating lipid compositions was much less for protein in 50 mM NaCl than for protein in 400 mM NaCl, suggesting that there is a difference in conformation at low and high NaCl concentrations. This suggestion is supported by the observation that binding of BoNT T domain to the LUV was weaker, as judged by the requirement for a several-fold higher concentration of lipid to give half-maximal quenching at high NaCl concentration than at low NaCl concentration.

However, as judged by far UV CD, which is sensitive to secondary structure, there is little effect of pH or lipid upon the secondary structure of the BoNT T domain, with perhaps a slight increase in helical structure at low pH in the presence of lipid relative to that at neutral pH (Fig. 4). This is consistent with the behavior of T-type domains from other toxins, which show only small increases in helix content upon insertion into membranes at low pH despite large pH- and/or lipid-induced changes in tertiary structure (Rosconi and London 2002; Zakharov et al. 1998, 1999).

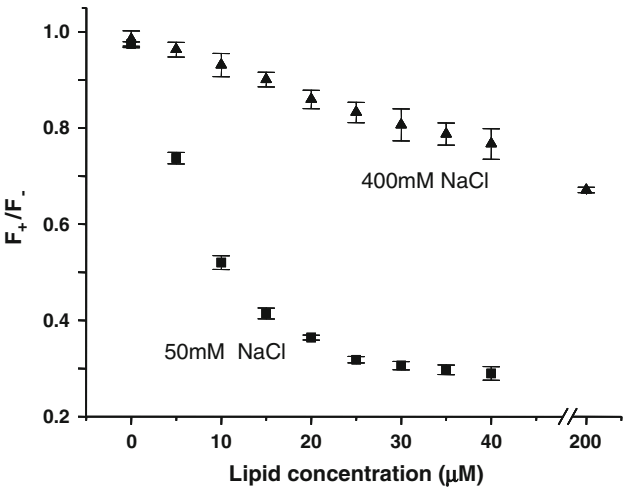

Fig. 3 Effect of lipid concentration upon the interaction of Cys-less BoNT T domain with LUV. Samples contained 20 μg/ml BoNT T and either (*squares*) 170 mM NaAc-50 mM NaCl (pH 4.0) or (*triangles*) 170 mM NaAc-400 mM NaCl (pH 4.0). Samples were titrated with aliquots of a stock solution of LUV containing 10 mM lipid. F_+/F_- is the ratio of fluorescence in the presence of vesicles containing 70 mol% DOPC/25 mol% DOPG/5 mol% pyrene-DOPE to that in the presence of vesicles composed of 70 mol% DOPC/30 mol% DOPG. The average value and range of duplicates are shown

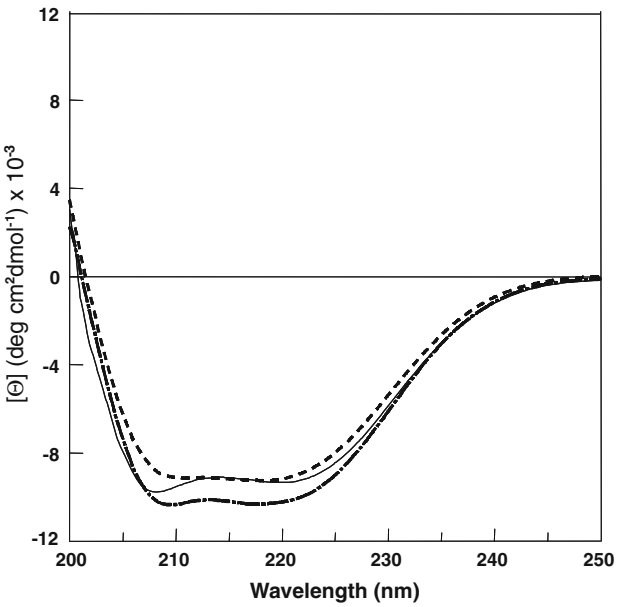

Fig. 4 Far UV CD spectra of BoNT T. BoNT T concentration was 0.2 mg/ml. Samples contained (*solid line*) 170 mM Tris–Cl-50 mM NaCl (pH 8), (*dashed line*) 170 mM NaAc 50 mM NaCl (pH 4) or (*dash–dot line*) 170 mM NaAc 400 mM NaCl (pH 4). Samples containing 170 mM NaAc-50 mM NaCl plus LUV or 170 mM NaAc-400 mM NaCl plus LUV composed of 200 μM 70% DOPC/30% DOPG gave spectra nearly identical to those in 170 mM NaAc 400 mM NaCl (not shown)

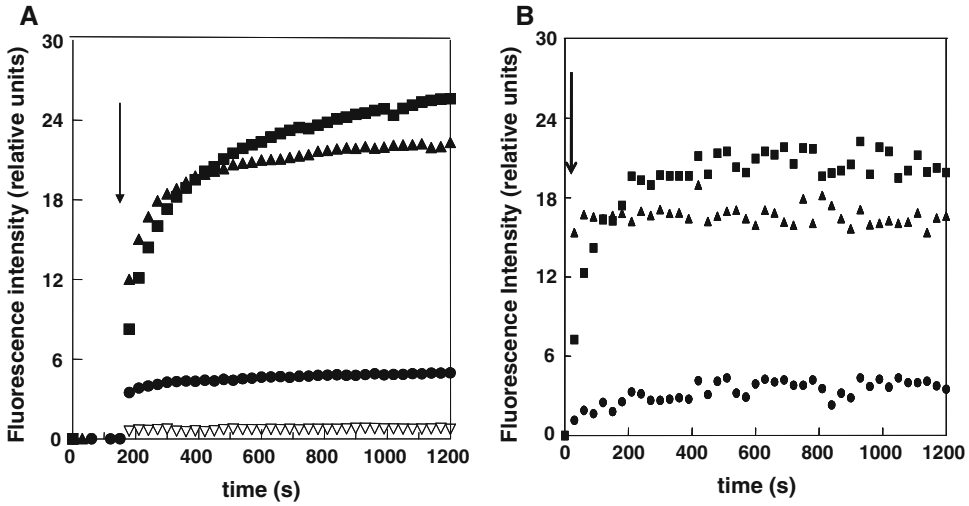

Fig. 5 Biocytin assay of pores formed by BoNT in LUV. **a** Cys-less BoNT T domain in LUV composed of 70 mol% DOPC/30 mol% DOPG. **b** Cys-less BoNT T domain in LUV composed of DOPC. Samples contained 200 nM (**a**) or 400 nM (**b**) BoNT T and 100 μM lipid with 66 ng/ml trapped BODIPY-SA and (*filled squares*) 170 mM NaAc-50 mM NaCl (pH 4.0), (*filled circles*) 170 mM NaAc-400 mM NaCl (pH 4.0) or (*open inverted triangles*) controls containing 170 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)–50 mM NaCl (pH 7.0). Samples were incubated with protein for 20 min. Then (time zero, shown by *arrow*) 20 nM biocytin was added. Fluorescence was measured every 30 s. In the experiments shown by *filled triangles* BoNT T domain was added to samples containing 170 mM NaAc–50 mM NaCl (pH 4.0) and after the 20-min incubation NaCl was increased to 400 mM by addition of an aliquot from 5 M NaCl. After 10 min further incubation, biocytin was added to assay the extent of pore formation. In **b** values for controls at pH 7, which show no increase upon BoNT T domain addition, were subtracted from the values shown. Average of duplicates is shown in **b**

The Effect of pH, NaCl Concentration and Protein Concentration upon Pore Formation by BoNT T Domain

Next, the extent of pore formation by BoNT T domain was examined using DOPG/DOPC vesicles. To do this the influx of the small molecule biocytin into vesicles was measured using vesicles with vesicle-entrapped BODIPY-SA. Upon influx of biocytin, it binds to BODIPY-SA and displaces the covalently bound BOPIPY group from the biotin binding site on the streptavidin molecule, which results in an increase in BODIPY fluorescence (Emans et al. 1995; Nicol et al. 1999; Rosconi et al. 2004). As shown in Fig. 5a, Cys-less BoNT T domain was able to form pores in LUV at low pH. The extent of pore formation was much greater at low NaCl concentration (50 mM) than at high NaCl concentration (400 mM). Similar results were obtained with wild-type T domains (Supplemental Fig. 1b). Increasing NaCl concentration to 400 mM after BoNT T domain had been incubated with vesicles at low pH and 50 mM NaCl did not result in a significant decrease in the apparent amount of pore formation (Fig. 5a).

Notice that in this uptake-based pore assay, the state of pore formation at the time that biocytin is added is detected. Thus, if pores were open at low salt and then became smaller or closed at high salt, this assay would have detected reduced pore formation. This would not be the case in a release-based pore assay, in which trapped material would be released when pores were large and subsequent pore closing would not be detected.

This result indicates that pore formation by inserted BoNT T at low salt cannot be decreased to that at high salt once the BoNT T domain has inserted. An alternative explanation, that at low salt BoNT pores are large enough to allow BODIPY-SA to escape the vesicles and thus remains reactive with biotin even though pore formation is reduced at high salt, is ruled out by chromatographic experiments which confirm that the pores formed by BoNT at low salt are not large enough to release trapped streptavidin (Supplemental Fig. 2).

The pH dependence of pore formation (Fig. 6) showed that pore formation is significant only at pH 5 and below. In the presence of a low salt concentration, pore formation increased as pH decreased, reaching a maximum value near pH 4.5 and then decreasing at lower pH values. At a high salt concentration, pore formation appeared to gradually increase as pH is decreased, but at no pH tested was the extent of pore formation as great in the presence of a high salt concentration as it was at a low salt concentration. At all low pH values, increasing salt concentration after BoNT T insertion did not decrease pore formation. The dependence of pore formation upon salt concentration at low pH (Fig. 7a) showed a very gradual decrease as salt concentration was increased.

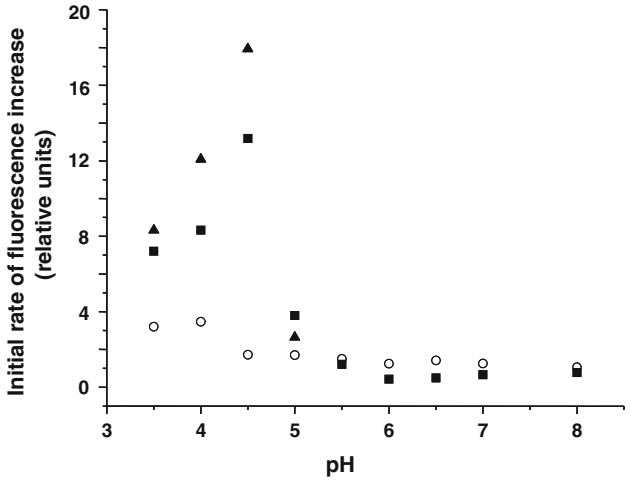

Fig. 6 pH-dependent pore formation by BoNT T at different NaCl conditions. Experimental conditions as in Fig. 5 except that samples were prepared at various pH values in 170 mM NaAc in the range pH 3.5–5.0, 170 mM MES in the range pH 5.5–6.5 or 170 mM Tris–Cl in the range pH 7.0–8.0. The increase in BODIPY fluorescence in the first 30 s after biocytin was added is shown on the *y* axis. Samples also contained (*squares*) 50 mM NaCl, (*circles*) 400 mM NaCl or (*triangles*) 50 mM NaCl increased to 400 mM NaCl 20 min after protein was added. (NaCl concentrations shown here and in the following figure are not corrected for NaCl in the LUV preparation, which slightly alters final NaCl concentration [by not more than 8 mM])

It has been reported that high salt concentrations reduce the degree of BoNT T domain insertion into membranes by interrupting electrostatic interactions between the protein and anionic lipids (Galloux et al. 2008). This could explain the reduced pore formation at high salt but predicts that when BoNT T domain is inserted at low salt and then salt concentration is increased, pore formation should decrease, which we did not observe. To further investigate this question, we examined the effect of salt concentration upon pore formation in vesicles composed solely of DOPC, a zwitterionic lipid. As shown in Fig. 5b, pore formation was greater when T domain inserted at low salt concentration than at high salt concentration. In addition, when T domain inserted at low salt concentration and then the salt concentration was increased, pore formation remained close to that at low salt concentration. These observations indicate that the effect of salt upon pore formation can involve an effect of salt upon the conformation of BoNT T domain, rather than, or in addition to, changes in the electrostatic interaction between protein and lipid.

The dependence of pore formation upon protein concentration (at low salt concentration) was also examined (Fig. 7b). There was a nonlinear dependence of pore formation upon BoNT concentration, with a large increase in the rate of pore formation at the highest concentration examined (~1 BoNT T domain molecule per 500 lipid molecules). This may mean that the size of the pore formed by BoNT T domain is dependent upon the concentration of the protein due to a concentration-dependent oligomerization (see "Discussion"). It should be pointed out that in these samples Trp fluorescence intensity was found to be linear in BoNT T concentration over the concentration range studied (not shown), indicating that the non-linear protein concentration dependence of pore formation was not an artifact of protein loss during sample preparation arising from the use of very dilute protein concentrations.

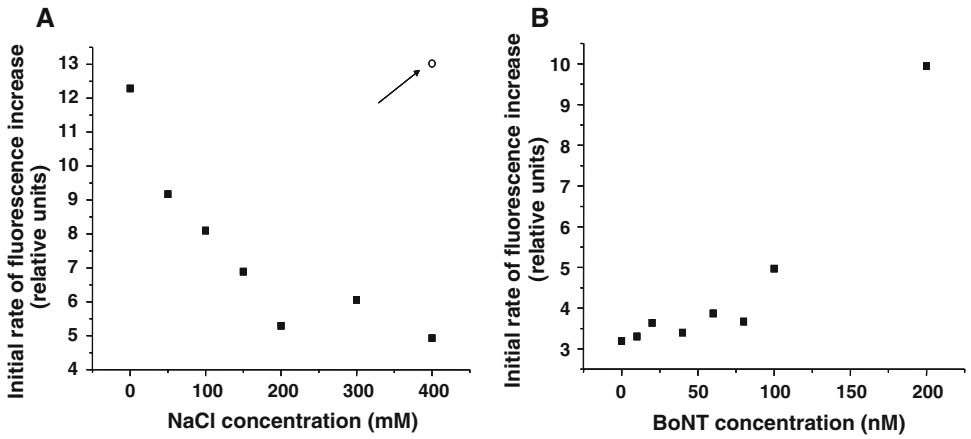

Fig. 7 Dependence of pore formation upon NaCl and BoNT T domain concentration. **a** Effect of NaCl concentration upon pore formation. (*filled squares*) Samples contained 200 nM BoNT T and LUV composed of 100 μM 70 mol% DOPC/30 mol% DOPG with 66 ng/ml entrapped BODIPY-SA dispersed in 170 mM NaAc (pH 4.0) and the NaCl concentration shown. The assay of pore formation was initiated by addition of 20 nM biocytin 20 min after BoNT T domain addition. The rate of biocytin influx after BoNT T was added (over the initial 30 s of uptake) is shown. Influx (*open circle*, indicated by *arrow*) in sample prepared at 50 mM NaCl but in which NaCl concentration was increased to 400 mM NaCl after 20-min incubation. The assay of pore formation was initiated 10 min after the NaCl concentration was increased. **b** BoNT T concentration-dependent pore formation. Samples prepared as described in **a** except BoNT T concentration was varied and NaCl concentration in the buffer used was 50 mM in all samples

Discussion

This report describes the properties of isolated BoNT T domain under different conditions. Because BoNT T has four Trps in various positions in its sequence, they are useful for probing general conformational changes, i.e., changes in overall tertiary structure. Changes in Trp fluorescence show that the isolated T domain undergoes a low pH-induced conformational change. At low pH, there is also a further change in Trp fluorescence upon interaction of BoNT T with lipid, accompanied by a blue shift in Trp fluorescence. This suggests that portions of the BoNT T domain penetrate the lipid bilayer, consistent with the induction of pore formation at low pH. These properties indicate that isolated BoNT T domain prepared in DDM maintains key functional properties previously observed in whole BoNT (Fu et al. 2002; Koriazova and Montal 2003; Montecucco et al. 1989; Puhar et al. 2004; Schiavo et al. 1990). This conclusion is reinforced by the similarity of the behavior of BoNT T to that previously observed for the isolated T domain of diphtheria toxin, which also is able to maintain a functional structure that can penetrate membranes and induce pore formation at low pH (Kachel et al. 1998; Sharpe and London 1999; Zhan et al. 1994), and is in agreement with the conclusions of a recent study of BoNT T domain by Galloux et al. (2008).

BoNT T domain also resembles the T domain of diphtheria toxin in that after membrane insertion at low pH BoNT T can exist in multiple conformations with different properties (as judged by the dependence of pore-forming and membrane interaction properties upon NaCl concentration). This knowledge is important because it is not possible to predict a priori what conditions should be used in structural studies carried out in vitro. In the absence of interactions that occur in intact protein under physiological conditions, the experimental conditions that seem closest to physiological conditions might actually result in the formation of a conformation that is not relevant to function. Indeed, in the case of diphtheria toxin T domain it is easy to choose experimental conditions that result in the formation of a membrane surface–associated conformation quite distinct from the deeply inserted conformation that is likely to be involved in catalytic chain translocation (Chenal et al. 2002; Wang et al. 1997b).

It should also be noted that there are significant structural differences between BoNT and diphtheria toxin T domains. The most striking is that the high-resolution solution structure of native diphtheria toxin T domain shows several well-defined hydrophobic transmembrane-like helices (Choe et al. 1992), while that of native BoNT T shows only two hydrophobic sequences which are not packed into well-defined classical transmembrane-like helices. Thus, the structure of membrane-inserted BoNT T is likely to be novel and may reveal a novel translocation motif/mechanism.

Therefore, the observation that salt concentration alters the behavior of BoNT T domain is likely to be important for future studies of BoNT T domain membrane topography. Low salt conditions, in which BoNT T domain shows the greatest degree of pore formation, are most likely to result in the formation of a membrane-inserted conformation relevant to translocation. Nevertheless, the state forming at higher salt concentrations may also represent a conformation involved in the translocation process, and its topography must also be characterized. The observation that the extent of pore formation is dependent upon the concentration of BoNT T may also be an important clue to the translocation mechanism. One possible explanation for such behavior is that BoNT T has the capacity to oligomerize and that pore size is dependent upon oligomer size, as has been observed for diphtheria toxin under some conditions (Sharpe and London 1999).

However, many questions remain concerning BoNT T domain pore properties. The pore formation experiments here do not distinguish between increased pore formation due to an enlarged pore size and increased number of pores. This will have to be examined in future studies. In addition, whether the effect of NaCl concentration involves an ion-specific interaction or simply an ionic strength effect is yet to be determined. [Previous studies detected pore formation by T domain in a solution containing 100 mM KCl rather than NaCl (Blaustein et al. 1987).]

Galloux et al. (2008) characterized the membrane interaction of BoNT T domain. In general, where that report and this one carried out similar experiments there is excellent agreement between them. However, our study adds important new information concerning the effect of salt concentration. First, the observation that increasing NaCl concentration decreases BoNT T domain pore formation in uncharged DOPC vesicles indicates that NaCl does not act solely by interfering with the electrostatic attraction between protein and anionic lipid vesicles identified by Galloux et al. Second, our observation that pore formation is not greatly reduced when salt concentration is increased in samples containing BoNT T domain membrane inserted at low salt concentration contrasts with the observation of Galloux et al. that increasing salt decreases T domain membrane penetration. One possible explanation is that key pore-forming segments of the T domain remain deeply inserted when salt concentration increases, while some Trp-bearing segments outside of the pore-forming domain move to a more shallow location.

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