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NOTE

Stimulation of Tissue Transglutaminase Activity by *Clostridium botulinum* Neurotoxin Type B

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Recombinant light chain of *Clostridium botulinum* neurotoxin type B stimulated transglutaminase activity in a dose dependent manner. Compared to native toxin, recombinant light chain showed a greater stimulatory effect on transglutaminase activity. Zn-chelating agents, inhibiting the proteolytic activity of the clostridial toxins, did not interfere with this stimulation. These results suggest that the light chain plays a major stimulatory role, which is not due to its metallopeptidase activity, but is possibly due to specific interaction with transglutaminase. More importantly, this report provides a new insight into the intracellular action of *C. botulinum* neurotoxins.

Key words: *Clostridium botulinum* neurotoxin type B, Zn-chelating agent, transglutaminase

The clostridial neurotoxins, a group of 150 kDa proteins, are produced by *Clostridium tetanus* and *Clostridium botulinum* (seven serotypes A to G), which cause tetanus and botulism by irreversibly blocking neurotransmitter release from presynaptic nerve endings via specific Zn-dependent protease activity (Montecucco and Schiavo, 1994). Each neurotoxin consists of a heavy chain (a 100 kDa C-terminus portion), which plays a role in the initial binding and internalization of the neurotoxins into nerve endings, and a light chain (a 50 kDa N-terminus portion), which acts intracellularly to block exocytosis (Humeau *et al.*, 2000). The light chain has metalloendopeptidase activity that cleaves a component of the exocytosis docking and fusion complex (Montecucco and Schiavo, 1993). Among these toxins, tetanus and botulinum toxin type B have been reported to cleave synaptobrevin (also called Vesicle-Associated-Membrane-Protein) at the Gln76-Phe77 position (Schiavo *et al.*, 1992).

Certain aspects of the actions of botulinum and tetanus toxin cannot be satisfactorily explained by the cleavage of some components that are necessary for the release of neurotransmitter in neuromuscular junction. One of these is the requirement of an intact cytoskeleton for the toxin to exert its full activity in synaptosomes (Ashton *et al*, 1993; Ashton and Dolly, 1997). Secondly, neurotransmitter release was found to be suppressed, even when the mRNAs of nonproteolytic mutants of the light chain were expressed in *Aplysia* neurons (Niemann, 1991; Yamasaki *et al.*, 1994). Moreover, it has also been reported that Zn-protease-deficient mutant tetanus toxin inhibited neurotransmitter release from *Aplysia* neurons, and that this inhibition was reduced when monodansylcadaverine, a transglutaminase inhibitor, was added (Ashton *et al.*, 1995). Overall, these results imply the hypothesis that the toxin blocks neurotransmitter release by the well-characterized cleavage of components of the exocytosis docking and fusion complex and possibly by another mechanism, involving transglutaminase.

Transglutaminases (R-glutaminyl-peptide:amine gammaglutamyltransferases, EC 2.3.2.13) are Ca^{++} and GTP-dependent enzymes that are present in different tissues, including the brain. These enzymes catalyze the formation of covalent bonds between protein-bound glutamine residues and primary amino-groups, resulting in protein cross-linking or in amine incorporation into proteins (Greenberg *et al.*, 1991). Moreover, the inhibitory activities of various amines, including monodansylcadaverine, on transglutaminase in synaptosomes correlates with their potencies at stimulating catecholamine release (Pastuszko *et al.*, 1986). It was, therefore, suggested that transglutaminase may be involved in the secretory events at the synapse, inhibiting neurotransmitter release. Moreover, it is

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also known that the nerve ending specific protein, synapsin I, is cross-linked by synaptosomal transglutaminase (Facchiano *et al.*, 1993a; 1993b), and that tetanus toxin potently binds to, and activates tissue transglutaminases *in vitro* (Facchiano and Luini, 1992).

In order to verify the hypothesis that transglutaminase is one of the most likely target proteins of botulinum toxin type B for the inhibition of neurotransmitter release, we investigated the effect of botulinum toxin type B on transglutaminase. The recombinant light chain of botulinum toxin type B was prepared from the *E. coli* strain JMDE-3BL21pLysS, containing the recombinant light chain expression vector, pETL1, using the procedure described by Rhee *et al.* (1997), and native BoNT/B was prepared as described by Evans *et al.* (1986). SDS-PAGE analysis demonstrated that both recombinant light chain and native BoNT/B were highly purified without any visible contaminant protein bands (data not shown). Commercial guinea pig liver transglutaminase (Sigma, USA) was purchased, and purified using a chromatography as reported previously (Facchiano and Luini, 1992).

The transglutaminase assay was carried out according to a published procedure (Takagi *et al.*, 1986), with minor modifications. Briefly, transglutaminase activity was measured by evaluating the incorporation of monodansylcadaverin into acetylated casein, at 37°C in 50 mM Tris-HCl (pH 7.8), 10 mM dithiothreitol, 2 mM $MgCl_2$, 1 mM $CaCl_2$. The enzyme reaction was stopped by the addition of 1 M ammonium sulfate to a final concentration of 42 mM. The fluorescence intensity of the mixture was measured using a Shimadzu RF5301PC fluorescence spectrophotometer equipped with a thermostatically controlled cell, at excitation and emission wavelengths of 350 and 480 nm, respectively. Furthermore, before measuring the toxin-induced effects, the mixture was pre-incubated for 5 min at 25°C without any enzyme substrate. The data obtained are presented as means±S.E., and the Dunnetts t-test was used to assess the significance of the treatment; significance was accepted at the $p<0.01$ level.

It has been reported that the recombinant light chain of BoNT/B, which shows high homology to tetanus toxin, cleaves nerve synaptobrevins as efficiently as the native botulinum toxin type B light chain (Rhee *et al.*, 1997). Based on the previous report that the recombinant light chain is fully active, we studied the effect of the recombinant light chain on transglutaminase activity. Fig. 1. shows that the recombinant light chain dose-dependently stimulated purified tissue transglutaminase. This is the first report to find that botulinum toxin stimulates transglutaminase activity.

The native botulinum toxin type B also stimulated tissue transglutaminase, under the reducing conditions resulting from dithiothreitol addition, demonstrating that the toxin can also stimulate transglutaminase under these such conditions (Table 1). The recombinant light chain elevated the activity of tissue transglutaminase more than the native toxin. However, the boiled recombinant light chain showed little stimulatory effect on transglutaminase activity. From these results, we presume that the light chain portion of the whole toxin may play a major role in stimulating tissue transglutaminase, which concurs with accepted opinion, namely, that the light chain mediates the intracellular toxification.

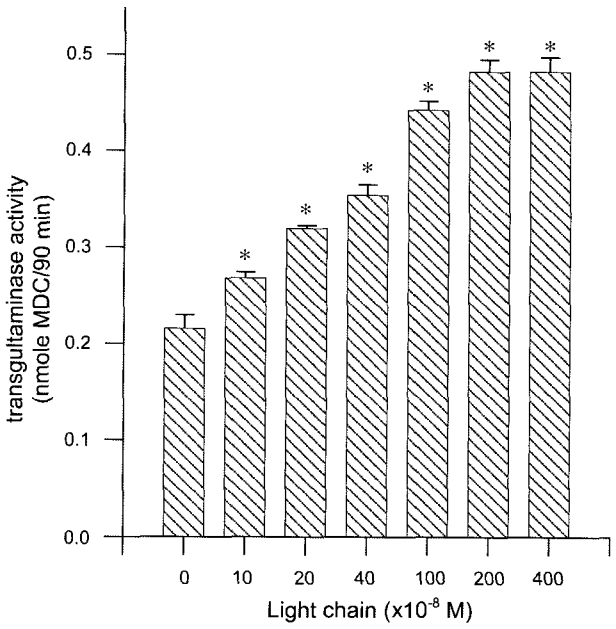

Fig. 1. Dose-dependant stimulatory effects of the recombinant light chain of botulinum toxin type B on transglutaminase activity. Purified guinea pig liver transglutaminase (20 nM) was pre-incubated with various concentrations of recombinant light chain for 5 min at 25°C, and transglutaminase activity was assessed. *Significantly different from control group ($p<0.01$).

Table 1. Stimulative effect of BoNT/B on transglutaminase

Transglutaminase stimulation (% of effect by BoNT/B)	
Native BoNT/B	100.00±2.26
Native BoNT/B+12 mM DTT	176.87±3.01*
Recombinant light chain	232.26±3.22*
Boiled recombinant light chain	22.06±5.32*

Guinea pig liver transglutaminase (10^{-7} M) was preincubated with native BoNT/B (10^{-7} M) or recombinant light chain (10^{-7} M) for 5 min at 25°C. The reaction was then carried out as described in text.
*Significantly different from native BoNT/B control ($p<0.01$).

Since the specific metalloprotease activity of the recombinant light chain has been well characterized (Rhee *et al.*, 1997), it may be that transglutaminase is stimulated by the proteolytic action of the recombinant light chain. Surprisingly, Table 2 shows that stimulation by the recombinant light chain was undiminished by certain specific metalloprotease inhibitors which efficiently inhibit recombinant

Table 2. Effects of Zn-protease inhibitors on recombinant light chain induced-transglutaminase stimulation

Transglutaminase activity (% of effect by recombinant light chain)	
Control	100±2.2
Recombinant light chain	163±5.5*
Recombinant light chain+EDTA	159±2.9*
Recombinant light chain+2,2-dipyridyl	163±3.7*
Recombinant light chain+Captopril	169±4.6*

Guinea pig liver transglutaminase (20 nM) and recombinant light chain (50 nM) were used for this experiment in the absence or presence of EDTA (40 mM), captopril (40 mM), or 2,2-di-pyridyl (80 mM).
*Significantly different from control ($p<0.01$).

light chain-induced synaptobrevin cleavage (Rhee *et al.*, 1997). Moreover, SDS-PAGE analysis showed that no transglutaminase cleavage occurred on treating with recombinant light chain (data not shown). It is thus expected that stimulation by the recombinant light chain may not be due to the proteolytic cleavage of transglutaminase, but rather a change in its catalytic activity, probably caused by a protein-protein interaction. The absence of a stimulatory effect of the boiled light chain on transglutaminase activity implies that the structural integrity of the light chain is necessary for transglutaminase stimulation by protein-protein interaction between BoNT/B and transglutaminase (Table 1). This hypothesis is supported by the finding that tetanus toxin produced no obvious cleavage of the purified tissue transglutaminase, although the toxin enhanced the enzymatic activity of transglutaminase (Coffield *et al.*, 1994).

The cleavage of synaptobrevin at a specific site by BoNT/B is well documented (Schiavo *et al.*, 1992), and this action certainly has a role in blocking neurotransmitter release in the cholinergic system. In addition to exocytosis inhibition due to the Zinc-dependent protease activity of botulinum toxin, the neurotransmitter blocking action of botulinum toxin may be partially caused by the inhibition of the dissociation of the synaptic vesicle from the cytoskeleton (Facchiano *et al.*, 1993a; 1993b). In neurons, it is well known that transglutaminase has a dominant function in the dissociation of synaptic vesicles from the cytoskeleton and in the breakdown of the cytoskeleton by cytochalasin D (a cholchicin that reduces the inhibition of exocytosis by tetanus toxin) (Ashton and Dolly, 1997).

Native botulinum toxin and the recombinant light chain play a role in the intracellular blocking action of neurotransmitter release, and in stimulating the tissue transglutaminase. Furthermore, the changes of transglutaminase activity may be caused by a mechanism other than the proteolytic activation process. These new findings might be helpful at elucidating the intracellular function of *C. botulinum* neurotoxins, and show them to be intracellular stimulators of transglutaminase.

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References

Ashton, A.C., A. de Pavia, B. Poulain, L. Tauc, and J.O. Dolly. 1993. Factors underlying the characteristic inhibition of the euronal release of transmitters by tetanus and various botulinum toxins, p.191-213. *In* B.R. DasGupta (ed.), Botulinum, tetanus neurotoxins: neurotransmission and biomedical aspects, Plenum Publishing Corp, New York.

Ashton, A.C. and J.O. Dolly. 1997. Microtubules and microfilaments participate in the inhibition of synaptosomal noradrenaline release by tetanus toxin. *J. Neurochem.* 68, 649-658.

Ashton, A.C., Y. Li, F. Doussau, U. Weller, G. Dougan, B. Poulain, and J.O. Dolly. 1995. Tetanus toxin inhibits neuroexocytosis even when its $Zn(2^+)$-dependent protease activity is removed. *J. Biol. Chem.* 270, 31386-31390.

Coffield, J.A., R.V. Considine, J. Jeyapaul, A.B. Maksymowych, R. Zhang, and L.L. Simpson, 1994. The role of transglutaminase in the mechanism of action of tetanus toxin. *J. Biol. Chem.* 269, 24454-24458.

Evans, D.M., R.S. Williams, C.C. Shone, P. Hambleton, J. Melling, and J.O. Dolly. 1986. Botulinum neurotoxin type B. Its purification, radioiodination and interaction with rat-brain synaptosomal membranes. *Eur. J. Biochem.* 154, 409-416.

Facchiano, F. and A. Luini. 1992. Tetanus toxin potently stimulates tissue transglutaminase. A possible mechanism of neurotoxicity. *J. Biol. Chem.* 267, 13267-13271.

Facchiano, F., F. Benfenati, F. Valtorta, and A. Luini, 1993a. Covalent modification of synapsin I by a tetanus toxin-activated transglutaminase. *J. Biol. Chem.* 268, 4588-4591.

Facchiano, F., F. Valtorta, F. Benfenati, and A. Luini, 1993b. The transglutaminase hypothesis for the action of tetanus toxin. *Trends Biochem. Sci.* 18, 327-329.

Greenberg, C.S., P.J. Birkbichler, and R.H. Rice, 1991. Transglutaminase: multifunctional cross-linking enzymes that stabilize tissues. *FASEB J.* 5, 3071-3077.

Humeau, Y., F. Dousaur, N.J. Grant, and B. Poulain. 2000. How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie.* 82, 427-446.

Montecucco, C. and G. Schiavo. 1993. Tetanus and botulism neurotoxins: a new group of zinc proteases. *Trends Biochem. Sci.* 18, 324-327.

Montecucco, C. and G. Schiavo. 1994. Mechanism of action of tetanus and botulinum neurotoxins. *Mol. Microbiol.* 13, 1-8.

Niemann, H. 1991. Molecular biology of clostridial neurotoxins, p.303-348. *In* J. Alouf and J. Freer, (ed.), A sourcebook of bacterial protein toxins, Academic Press, London.

Pastuszko, A., D.F. Wilson, and M. Erecinska, 1986. A role for transglutaminase in neurotransmitter release by rat brain synaptosomes. *J. Neurochem.* 46, 499-508.

Rhee, S.D., H.H. Jung, G.-H. Yang, Y.S. Moon, and K.-H. Yang, 1997. Cleavage of the synaptobrevin/vesicle-associated membrane protein (VAMP) of the mouse brain by the recombinant light chain of Clostridium botulinum type B toxin. *FEMS Microbiol. Lett.*, 150, 203-208.

Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, P. Polverino de Laureto, B.R. DasGupta, and C. Montecucco. 1992. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature*. 359, 832-835.

Takagi, J., Y. Saito, T. Kikuchi, and Y. Inada. 1986. Modification of transglutaminase assay: use of ammonium sulfate to stop the reaction. *Anal. Biochem*. 153, 295-298.

Yamasaki, S., Y.G. Hu, T. Binz, A. Kalkuhl, H. Kurazono, T. Tamura, R. Jahn, E. Kandel, and H. Niemann. 1994. Synaptobrevin/vesicle-associated membrane protein (VAMP) of Aplysia californica: structure and proteolysis by tetanus toxin and botulinal neurotoxins type D and F. *Proc. Natl. Acad. Sci U.S.A*. 91, 4688-4692.