Volume 261, number 2, 323–326 FEBS 08158 February 1990

Ca^{2+}-dependent noradrenaline release from permeabilised PC12 cells is blocked by botulinum neurotoxin A or its light chain

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Received 12 January 1990

Permeabilisation of PC12 cells with digitonin allowed a direct study of the intracellular action of botulinum neurotoxin A, one of a group of dichain proteins produced by *Clostridium botulinum* that causes the fatal neuroparalytic condition, botulism. Release of [^{3}H]noradrenaline from these permeabilised cells could be evoked by Ca^{2+} and this was inhibited specifically by the neurotoxin in a dose-dependent manner (half-maximal dose ~2 nM under the conditions used). Inclusion of the reducing agent dithiothreitol (up to 10 mM) had no effect on the level of inhibition. Moreover, electrophoretic analysis showed that this treatment of the toxin in the native state caused negligible reduction of inter-chain disulphide bonds. Toxin-induced blockade of neurotransmitter release was incomplete and could not be overcome by increased Ca^{2+} concentration (100 μM). The observed toxin-insensitivity of the release from intact PC12 cells must result from inefficient toxin uptake, relative to that in peripheral cholinergic neurones. Refolded light chain alone inhibited exocytosis to the same degree and with similar potency to that of the intact neurotoxin, an effect not altered by the heavy chain. This inhibitory activity of the light chain in PC12 cells accords with observations made in permeabilised chromaffin cells [(1989) J. Biol. Chem. 264, 10354–10360; (1989) FEBS Lett. 255, 391–394] but contrasts with invertebrate neurones, where intracellular injection of the same preparations of both chains were necessary for inhibition of quantal release of acetylcholine [(1988) Proc. Natl. Acad. Sci. USA 85, 4090–4094]. These collective findings may signify an interesting difference in the release process in such diverse systems or denote a dissimilarity in the transport or processing of the toxin when applied into intact neurones or cells permeabilised by detergent or streptolysin.

Botulinum neurotoxin; Noradrenaline release; Permeabilisation; Exocytosis; (PC12 cells)

1. INTRODUCTION

Botulinum neurotoxin (BoNT) type A is one of a family of closely-related proteins (M_r ~150000) produced by different strains of *Clostridium botulinum* (reviewed in [1]). Its unique toxicity results from a remarkably potent blockade of acetylcholine release from peripheral nerve endings [2]. Although BoNT binding is specific to cholinergic nerves [3], a prerequisite for its internalization [4] and action [5], secretion in other cells can be blocked when toxin uptake is bypassed [5–7]. BoNT is synthesised as a single chain, but only becomes fully toxic following proteolytic processing to a disulphide linked dichain form, composed of a heavy (M_r ~100000; HC) and a light (M_r ~50000; LC) chain [1,2]. The individual chains can be separated and, after refolding, each is relatively non-toxic in mice and ineffective on neuromuscular transmission [8]. Moreover, when injected separately into *Aplysia* neurons no alteration in transmitter release ensues; however, nerve-evoked release of aceetylcholine is inhibited when **both** chains are administered [5].

Because intraneuronal action of the chains cannot be tested directly in vertebrate motor nerves, PC12 cells were used here to ascertain if both polypeptides are *generally* required intracellularly for the blockade of Ca^{2+}-evoked neurotransmitter release. This is particularly pertinent because LC of BoNT [9,10] or tetanus toxin [11,12] were shown recently to block exocytosis from chromaffin cells. PC12 cells offer the advantage of being a relatively homogeneous population of neurone-like cells which are convenient for measurement of secretion, before and after differentiation [13]. Moreover, cell permeabilisation with digitonin [9,11] or pore-forming toxins [10,12] allows constituents in the intracellular milieu to be altered and, also, affords access of the toxin to its pharmacological target, whilst enabling quantitation of exocytosis. Another facet addressed herein is whether treatment of BoNT A with DTT increases its potency in blocking exocytosis as reported for streptolysin-permeabilised [14] but not digitonin-treated chromaffin cells.

2. EXPERIMENTAL

2.1. *Materials*

Homogeneous BoNT A was purified from *Clostridium botulinum* (strain NCTC 7272) to a specific toxicity of 2×10^8 mouse LD_{50} units/mg and its two chains were isolated as described previously,

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Abbreviations: BoNT, botulinum neurotoxin; Pipes, 1,4-piperazine-diethane sulphonic acid; Kgep, K^+ glutamate/EGTA/Pipes buffer; Hepes, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid]; DTT, dithiothreitol; LC, light chain; HC, heavy chain

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00145793/90/$3.50

with their purity being ascertained by SDS polyacrylamide gel electrophoresis [8]. The individual chains, obtained in buffer containing DTT and urea, were renatured by repeated dialysis, with agitation at 4°C for a minimum of 4 h, into Kgep (139 mM K^+ glutamate/20 mM Pipes/5 mM EGTA/1 mM Mg^{2+}-ATP/5 mM glucose/0.5 mM ascorbic acid, pH 6.6). The preparations were stored at 4°C; any insoluble material was removed by centrifugation and protein concentrations of the chains were determined [15]. Antibodies against BoNT A were raised in rabbits as detailed elsewhere [16]; 1 ml of the antiserum neutralised 10^7 mouse LD_{50} units of BoNT A.

2.2. *Permeabilisation of PC12 cells and measurement of noradrenaline release*

PC12 cells were grown on 100 mm dishes in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) horse serum, 5% (v/v) foetal calf serum, 3 mM glutamine and including penicillin (100 IU/ml) and streptomycin (100 μg/ml) (all from Flow Laboratories Ltd., Rickmansworth, England). Cells were plated at 2 × 10^3 cells/mm^2 on polylysine-coated, 24-well multi-wells (Falcon Labware, Marathon Laboratory Supplies, London) 1 or 2 days prior to use. Intact cells were incubated in the absence or presence of BoNT, washed, loaded with [^{3}H]noradrenaline and the levels of Ca^{2+}-dependent resting and depolarisation-induced release of transmitter from replicate wells was quantified as detailed in fig.1.

For permeabilisation experiments, cells were loaded with [^{3}H]noradrenaline and washed, as detailed for the intact cells (fig.1 legend). Optimal permeabilisation conditions were: 7.5 μM digitonin (Fluka Chemicals Ltd., Glossop, England) in 250 μl of Kgep for 5 min at room temperature [9]. Following removal of the medium, the quantities of [^{3}H]noradrenaline and lactate dehydrogenase (a cytoplasmic marker) released on permeabilisation were determined. Cells were then treated with the desired concentration of BoNT A in 250 μl of Kgep containing 1 mg/ml albumin and 1 mM NAD^+ for 15 min at 30°C. Neurotransmitter release was stimulated for 5 min at room temperature, with 10 μM free Ca^{2+} calculated by a computer programme [17] to require the addition of 4.43 mM $CaCl_2$ to the Kgep buffer with albumin. Ca^{2+}-independent release was measured similarly but with the omission of Ca^{2+} from the buffer. All samples were counted at an efficiency of 35% in scintillation cocktail (3:1 xylene/Triton, 0.3% PPO). The radioactive content of the cells was determined, likewise, after solubilization in 250 μl of 1% (v/v) Triton X-100 for 5 min.

2.3. *SDS polyacrylamide gel electrophoresis*

Gels of 10% acrylamide were cast and samples prepared as described previously [8].

3. RESULTS

Release of [^{3}H]noradrenaline by intact PC12 cells induced by depolarization with high concentrations of K^+ was highly dependent upon Ca^{2+} in the medium (Fig. 1A). Exposure to BoNT A failed to inhibit either the resting or the K^+-evoked release of noradrenaline from the cells (Fig. 1A), presumably due to a lack of toxin uptake because of the known absence of BoNT A binding sites on PC12 cells (A.C. Ashton and J.O. Dolly, unpublished data). To overcome this, the cells were treated with digitonin under conditions that gave permeabilisation of the plasma membrane without causing appreciable leakage of vesicular [^{3}H]noradrenaline. The ability to release noradrenaline in response to Ca^{2+} (10 μM free concentration) was retained after this treatment (fig.1B). BoNT A did not lower the Ca^{2+}-independent efflux; in contrast, it greatly reduced the Ca^{2+}-dependent transmitter release from

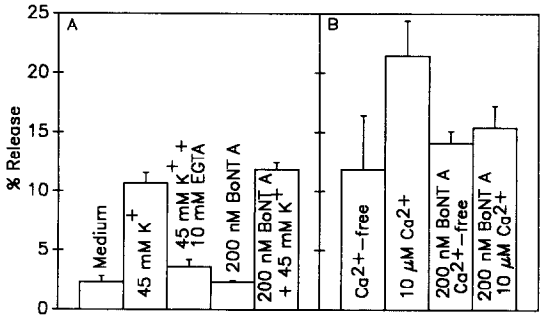

Fig.1. The effect of BoNT A on neurotransmitter release from non-permeabilised and permeabilised PC12 cells. Intact cells (A) were incubated at 37°C overnight in 250 μl of medium with or without 200 nM BoNT A. This was replaced with 250 μl of medium containing 0.4 μCi [7,8-^{3}H]noradrenaline (15 Ci/mmol, Amersham International, Bucks) for 1.5 h at 37°C. After three washes with medium containing 25 mM Hepes, pH 7.4, intact cells were incubated at room temperature for 5 min without any additions, with 45 mM KCl or with 45 mM KCl and 10 mM EGTA. Permeabilised (see section 2) cells (B) were treated for 15 min at 30°C with KGEP (+ albumin/NAD^+) with or without 200 nM BoNT A, followed by a 5 min incubation with KGEP (+ albumin) with or without 10 μM free Ca^{2+}. The amounts of radioactivity released were quantified by scintillation counting and expressed as % of the cellular content (in the case of permeabilised cells after digitonin and toxin treatment). Points (± SD) presented are from 4 independent wells for each condition and are representative of at least 10 experiments.

the permeabilised cells. Notably, toxin treatment failed to completely abolish the efflux (fig.1B; see below). The specificity of the blockade produced by BoNT was ascertained by loss of the effects seen when antiserum against the toxin was included (fig.2). Stimulation with high concentrations of free Ca^{2+} (up to 100 μM) produced a further (~20%) increase in release indicating that the standard concentration used (10 μM) was close to optimal; however, the raised level of Ca^{2+} did not overcome the BoNT-induced blockade (fig.2). BoNT A intoxication was temperature-dependent with much reduced effect on release being evident at room temperature (not shown).

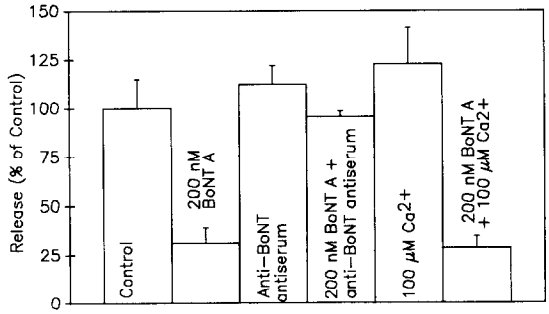

Fig.2. The specific reduction of Ca^{2+}-evoked noradrenaline release is not reversed by increasing the Ca^{2+} concentration. Cells permeabilised as in fig.1B were treated with buffer, BoNT A, anti-BoNT antiserum or BoNT A plus anti-BoNT antiserum as illustrated. Release was evoked by 10 or 100 μM free Ca^{2+}; Ca^{2+}-independent release was subtracted and the Ca^{2+}-dependent release expressed as a % of the control value. The results presented (± SD) are the means of data from 16 wells with and 16 without Ca^{2+}.

BoNT blockade of noradrenaline release was dose-dependent (fig.3) with a half-maximal effect at ~2 nM. Higher concentrations of BoNT (up to 1 μM) did not lead to further reduction in the level of release (not shown); therefore, there exists a BoNT A-insensitive fraction of Ca^{2+}-evoked transmitter release. Adding DTT (10 mM) did not alter the evoked release from the cells and likewise caused no significant increase in the effectiveness of a sub-optimal concentration of BoNT A in blocking secretion (fig.4A).

The individual chains of BoNT A were isolated as described previously and shown to be homogeneous by SDS polyacrylamide gel electrophoresis (see [8]); following refolding of the separate chains, each preparation was found to have low toxicity when injected into mice ($>10^5$-fold lower than that of intact BoNT). Moreover, neither chain (30 nM) produced any blockade of neurotransmission in mouse hemidiaphragm over 6 h [8]. When added to the permeabilised cells LC alone yielded a reduction in noradrenaline release comparable to that of the native toxin (figs 3 and 4). HC (100 μM) had no measurable effect on transmitter release; furthermore, inclusion of 100 nM HC with 2 nM LC did not increase the inhibitory activity of this sub-optimal concentration of LC alone (fig.4B).

The ability of varying concentrations of DTT were tested electrophoretically for their ability to reduce the toxin's interchain disulphide bonds under non-denaturing conditions. Following a brief incubation with DTT in the KGEP buffer, excess iodoacetamide was added to the mixture to prevent further reduction of disulphides exposed on denaturation of the toxin with SDS. The protein was then subjected to SDS polyacrylamide gel electrophoresis (fig.5). There was no detectable reduction of the disulphide bridge between the two polypeptide chains of the *native* toxin

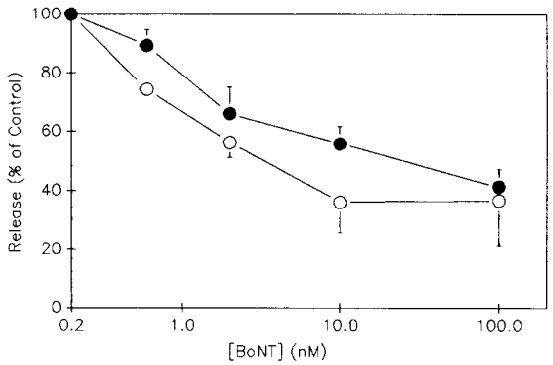

Fig.3. Dose dependence of the inhibition by BoNT A or its LC of Ca^{2+}-dependent release of [^{3}H]noradrenaline from permeabilised PC12 cells. After loading, washing and treatment with digitonin, the cells were incubated with varying concentrations of BoNT (●) or light chain (○), prior to measurement of release as detailed in section 2 and fig.1. Release is expressed as a percentage of the control, in the absence of toxin preparation. Points (± SD) are for 8 wells with and 8 without Ca^{2+}.

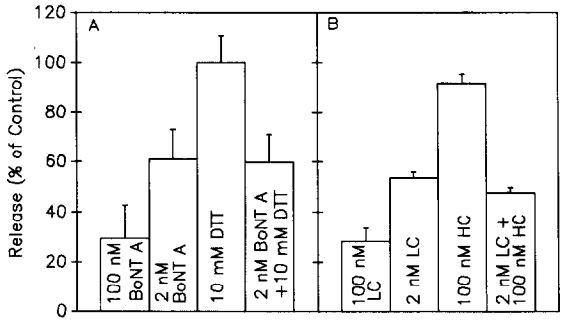

Fig.4. Reduction of noradrenaline release from permeabilised PC12 cells by BoNT or its chains. Cells were permeabilised as before and then treated as illustrated in A and B. Release was evoked as described earlier with or without 10 μM free Ca^{2+} and Ca^{2+}-dependent release expressed as a % of the control. Data (± SD) are the mean of 16 with and 16 without Ca^{2+}.

with 1 mM DTT when iodoacetamide was included after the initial reduction; similar treatment with 10 mM DTT caused cleavage of a small proportion of the molecules into their constituent chains (fig.5). When iodoacetamide was omitted some separation of the chains was seen with 1 mM DTT and this was more pronounced with the higher concentration used, indicating that unfolding of the toxin with SDS is needed to allow reduction to occur.

4. DISCUSSION

The ability of digitonin-permeabilised PC12 cells to release transmitter in a Ca^{2+}-dependent manner was exploited to investigate the intracellular inhibitory action of BoNT and its chains. Native BoNT produced an ex-

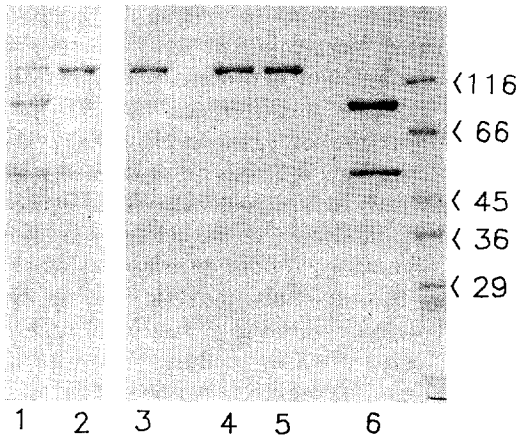

Fig.5. SDS polyacrylamide gel electrophoresis of BoNT A treated with DTT or iodoacetamide. Samples of BoNT A (5 μg) were treated with 1 mM, with 10 mM or without DTT as shown. After 10 min at room temperature, iodoacetamide (5 × the DTT concentration) was then added to prevent further reduction. Lanes are 1, 10 mM DTT; 2, 10 mM DTT + iodoacetamide; 3, 1 mM DTT; 4, 1 mM DTT + iodoacetamide; 5, BoNT A only; 6, BoNT A boiled in 2-mercaptoethanol. Molecular mass standards were: 116 kDa, *E. coli* β-galactosidase; 66 kDa, bovine albumin; 45 kDa, egg albumin; 36 kDa, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase; 29 kDa, bovine erythrocyte carbonic anhydrase.

tensive inhibition (60–70%) of Ca^{2+}-evoked release of noradrenaline from the permeabilised cells, consistent with the intracellular inhibition of exocytosis in chromaffin cells revealed either by measuring release directly [9,14] or membrane capacitance changes [6]. The half-maximal concentration of the toxin (~2 nM) in PC12 cells approximates to the values reported for chromaffin cells [9,14] and to the concentration (5 nM) effective intracellularly over 1–2 h in *Aplysia* neurones [5]. Although a lower toxin concentration has been found to be active following bath application of BoNT to mammalian motor nerves [8], any apparent discrepancy is likely to arise from the shorter time (15 min) of exposure to BoNT (enforced by the experimental system) rather than from a difference in the toxin's potency on different transmitters because a similar blockade of evoked quantal release has been observed in cholinergic and non-cholinergic neurones of *Aplysia* [5]. Treatment of the toxin with DTT did not improve the potency, in agreement with results obtained with chromaffin cells permeabilised under the same conditions [9]. As the DTT does not break the interchain disulphide bridge of BoNT A (fig.5), the apparent enhancement of intoxication seen in streptolysin O-permeabilised chromaffin cells [14] may arise from reduction of intradisulphide bonds; although this effect may be a feature of the latter cell system it is curious that reduction enhanced type B neurotoxin-mediated inhibition in digitonin-treated chromaffin cells [9].

Notably, BoNT-induced inactivation of the release process was achieved in Ca^{2+}-free medium containing EGTA; thus, the reported involvement of Ca^{2+} [18] must relate to another step of the intoxication (e.g. toxin transport to the cytosol). Moreover, the increased Ca^{2+} concentration failed to reverse the inhibition in the toxin-treated, permeabilised cells; the reversibility seen at the mammalian neuromuscular junctions (cf. [2]), could indicate a difference between the two systems or, more probably, incomplete inactivation in the latter preparation. Whereas nerve-evoked release at motor nerve terminals is virtually abolished by BoNT A treatment [1,2], inhibition in the cells was incomplete, even at the highest toxin concentration tested (1 μM). Nevertheless, this anomaly may be due to the experimental conditions used (e.g. restricted time of exposure to toxin; loss of cellular constituents on permeabilisation; incomplete permeabilisation of a proportion of the cells allowing Ca^{2+} entry but not affording access to the much larger toxin molecule) or could reflect two separate pools of neurotransmitter.

Isolated, re-natured LC inhibited neurotransmitter release from the digitonin-permeabilised PC12 cells in a dose-dependent fashion and with similar efficacy to the intact toxin (fig.3); and it approximates to LC-treated chromaffin cells [9,10]. Inclusion of excess isolated, renatured HC with LC failed to increase this level of inhibition. Interestingly, and by contrast, isolated LC of tetanus toxin can inhibit neurotransmitter release from either permeabilised chromaffin cells [19] or when micro-injected into *Aplysia* neurones [20]. Thus, further experiments with mammalian neurones are needed to establish if these differences mirror evolutionary changes in the mechanism of Ca^{2+}-dependent secretion, or if the method of noradrenaline release by endocrine-derived cells is distinct from that by neurones; alternatively, these findings might be explained by a requirement of the transport of LC when injected into *Aplysia* neurones. At least variations in the isolation or refolding of the toxin's chains have been excluded because identical results were obtained in the PC12 cells and *Aplysia* neurones with the same preparations of LC and HC; as well as samples kindly provided by Dr B.R. Dasgupta.

Acknowledgements: This work is supported by USAMRIID (Contract No. DAMD17-88-C-8008) and AFRC. PC12 cells were kindly provided by Dr D. Cutler in this department. We thank Professor J. Melling and Dr C. Shone (PHLS, Porton Down) for their continued support in the preparation of toxin, and Dr B.R. Dasgupta (Food Research Institute, Wisconsin, USA) for the provision of toxin chains.

REFERENCES

[1] Dolly, J.O., Poulain, B., Maisey, E.A., Wadsworth, J.D.F., Ashton, A.C., Breeze, A.L. and Tauc, L. (1988) in: Toxins in Neurochemistry (Dolly, J.O. ed.) pp.79–99, Ellis Horwood, Chichester, England.
[2] Sellin, L.C. (1987) Asia Pacific J. Pharmacol. 2, 203–222.
[3] Black, J.D. and Dolly, J.O. (1987) Neuroscience 23, 767–779.
[4] Black, J.D. and Dolly, J.O. (1986) J. Cell Biol. 103, 535–544.
[5] Poulain, B., Tauc, L., Maisey, E.A., Wadsworth, J.D.F., Mohan, P.M. and Dolly, J.O. (1988) Proc. Natl. Acad. Sci. USA 85, 4090–4094.
[6] Penner, R., Neher, E. and Dreyer, F. (1986) Nature 324, 76–78.
[7] Knight, D.E. (1986) FEBS Lett. 207, 222–226.
[8] Maisey, E.A., Wadsworth, J.D.F., Poulain, B., Shone, C.C., Melling, J., Gibbs, P., Tauc, L. and Dolly, J.O. (1988) Eur. J. Biochem. 177, 683–691.
[9] Bittner, M.A., Dasgupta, B.R. and Holz, R.W. (1989) J. Biol. Chem. 264, 10354–10360.
[10] Stecher, B., Weller, U., Habermann, E., Gratzl, M. and Ahnert-Hilger, G. (1989) FEBS Lett. 255, 391–394.
[11] Bittner, M.A., Habig, W.H. and Holz, R.W. (1989) J. Neurochem. 53, 966–968.
[12] Ahnert-Hilger, G., Weller, U., Dauzenroth, M.-E., Habermann, E. and Gratzl, M. (1989) FEBS Lett. 242, 245–248.
[13] Green, L.A. and Tischler, A.S. (1982) Adv. Cell Neurobiol. 3, 373–414.
[14] Stecher, B., Gratzl, M. and Ahnert-Hilger, G. (1989) FEBS Lett. 248, 23–27.
[15] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[16] Ashton, A.C., Crowther, J.S. and Dolly, J.O. (1985) Toxicon 23, 235–246.
[17] Howell, T.W. and Gomperts, B.D. (1987) Biochim. Biophys. Acta 927, 177–183.
[18] Simpson, L.L. (1980) J. Pharmacol. Exp. Ther. 212, 16–21.
[19] Ahnert-Hilger, G., Weller, U., Dauzenroth, M.-E., Habermann, E. and Gratzl, M. (1988) FEBS Lett. 242, 245–248.
[20] Mochida, S., Poulain, B., Weller, U., Habermann, E. and Tauc, L. (1989) FEBS Lett. 253, 47–51.