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Light chain of botulinum neurotoxin is active in mammalian motor nerve terminals when delivered via liposomes

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Liposomal encapsulation of the individual light and heavy chain of botulinum neurotoxin A was used to investigate their intra-cellular effects on synaptic transmission at the murine neuromuscular junction. Bath-application to phrenic nerve-hemidiaphragms of liposomes containing heavy chain (up to 75 nM) caused no alteration in neurally-evoked muscle tension. In contrast, liposomes with entrapped light chain (9–20 nM final concentration) gave a pre-synaptic blockade of neuromuscular transmission that could be relieved temporarily by 4-aminopyridine, as for the di-chain toxin. Any contribution from contaminating intact toxin was excluded both by the purity and minimal toxicity in mice of the light chain preparations used, and by the lack of neuromuscular paralysis seen with liposomes containing the maximum amount of native toxin that could have been present in the light chain liposomes. As bath-application of high concentrations of light chain in the absence of liposomes failed to affect neurotransmiter release, it is concluded that this chain alone can mimic the action of the whole toxin inside mammalian motor nerve endings, its predominant site of action. Thus, light chain could provide a more effective probe for an intra-cellular component concerned with Ca^{2+}-dependent secretion.

Botulinum neurotoxin; Light chain; Liposomal entrapment; Transmitter release; Neuromuscular junction

1. INTRODUCTION

Botulinum neurotoxin A (BoNT), one of a family (types A–G) of high molecular weight proteins ($M_r \approx 150\,000$) produced by strains of *Clostridium botulinum* (reviewed in [1]), preferentially and irreversibly inhibits the release of acetylcholine (ACh) in the peripheral nervous system [2]. The active di-chain form of the toxin consists of a heavy chain (HC; $M_r \approx 100\,000$) and a light chain (LC; $M_r \approx 50\,000$) joined by at least one disulphide bond [1]. Intoxication appears to involve a tri-phasic pathway [2]: (i) toxin binding to a specific ecto-acceptor on the pre-synaptic membrane – an innocuous process; (ii) an internalisation step with a relatively high Q_{10}; and (iii) an intracellular event that inhibits neurotransmitter release. Although this model has been documented by experimentation in various cell systems [3–7] an intraneuronal action of BoNT or its chains has not yet been demonstrated at motor endplates.

For optimal activity of BoNT at the neuromuscular junction the di-chain molecule is required; removal of the C-terminal (H_1) half of the HC abolishes its neuroparalytic activity in vitro [8]. Likewise, the renatured, individual chains of BoNT are ineffective in blocking neuromuscular transmission, and a mixture of HC and LC inhibits with relatively low potency [9]. However, in *Aplysia californica* the HC (or the N-terminal moiety (H_2)) can mediate acceptor-dependent translocation of LC [4,8] indicating a less stringent structural requirement for toxin uptake. When applied intra-cellularly to chromaffin cells [5,6] or added to permeabilised PC-12 cells [7], LC alone does inhibit Ca^{2+}-evoked noradrenaline release. In contrast, in *Aplysia*, inhibition of quantal release of neurotransmitter by intra-neuronally administered LC does not occur until HC is micro-injected inside or added to the bath [4]. A further anomaly is that the LC of tetanus toxin, a related *Clostridial* protein, blocks neurotransmitter release when injected in *Aplysia* [10].

Hence, it is important to establish if LC of BoNT is active inside mammalian motor nerve terminals, its prime target. As the permeabilisation techniques used for cultured cells [5–7] are not applicable to this preparation, in the present study BoNT or its chains were delivered via liposomes and their effects on ACh release were assessed by measuring changes in nerve-evoked muscle tension.

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Abbreviations: BoNT, botulinum neurotoxin A; LC and HC, light and heavy chain; ACh, acetylcholine; PC, phosphatidyl choline; PS, phosphatidyl serine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulphonic acid); 4-AP, 4-aminopyridine; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

2. EXPERIMENTAL

2.1. *Materials*

Egg phosphatidyl choline (PC) and phosphatidyl serine (PS) were obtained from Lipid Products (South Nutfield, Surrey), cholesterol

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from Sigma Chem. Co. (Poole, Dorset); Sephacryl S200 HR from Pharmacia Ltd. (Milton Keynes, Bucks.).

2.2. *Preparation of LC and HC*

BoNT was purified to homogeneity as detailed previously [11]. For preparations containing significant proportions of the single-chain species, the di-chain form was created by limited proteolysis in 50 mM triethanolamine/HCl buffer, pH 7.7, with Arg C (20 units/mg protein) at 37°C for 60 min. After arresting the digestion with tosyl-L-lysine chloromethyl ketone (2 mM), LC and HC were isolated chromatographically using an established procedure [9]. The purity of the chains was ascertained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and mouse toxicity. As the toxicity of the constituent chains is many orders of magnitude lower than the native toxin ($\approx 10^8$ LD_{50}/mg), the mouse bioassay provided a sensitive method of determining any contamination by the native toxin, that would be undetectable by silver staining of SDS-PAGE gels. LC and HC were renatured by dialysis into 118 mM NaCl/10 mM Hepes, pH 7.4, over 5 h (with 3 changes of buffer) at 4°C before preparing liposomes.

2.3. *^{125}I-Labelling of BoNT, LC and HC*

BoNT type A was radioiodinated to a high specific activity ($\approx$ 500 Ci/mmol) as described elsewhere [12] and the iodinated chains separated using the protocol referred to above and gave specific activities of $\approx$ 100 Ci/mmol and $\approx$ 400 Ci/mmol, respectively.

2.4. *Liposome preparation*

Liposomes were prepared using a modification of a previously described method [13]. Chloroform solutions of PC/cholesterol/PS in a 7:2:1 ratio (w/w) were mixed and dried under vacuum. The lipids were suspended in 4 ml of 118 mM NaCl/10 mM Hepes, pH 7.4, containing LC, HC or BoNT (0.25 mmol lipid/μmol protein; the approximate M_r of PS and PC was taken as 850). Because of the need to use minute amounts of BoNT (see below), heat-inactivated bovine serum albumin was added to the lipid suspension to adjust the total protein content to that of the LC or HC used in the preparation of liposomes. Trace amounts of the respective iodinated chains or BoNT were included to allow quantitation of their subsequent entrapment by liposomes. The lipid/protein mixtures were vortexed repeatedly over 30 min at 4°C before sonicating for 1 min with a MSE probe sonicator. After standing for 30 min at 4°C, the mixtures were loaded onto a Sephacryl S200 HR column (2.5 × 20 cm) previously equilibrated in the NaCl/Hepes buffer at 4°C. The liposome peak (6–8 ml) was detected by measuring turbidity (A_{600nm}) of the fractions collected and the protein quantified by counting radioactivity. The pooled liposome peak was dialysed over 14–16 h at 4°C into Krebs solution of the following composition (mM): Na^+ 143.0, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, Cl^- 127.7, HCO_3^- 25.0, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, glucose 11.1, pH 7.4. Samples were aerated (95% O_2/5% CO_2) before applying to the nerve-diaphragm preparations.

2.5. *Measurement of neuromuscular transmission*

Left phrenic nerve hemidiaphragms were dissected from Balb/C mice (20–25 g) and transferred immediately to a closed circulating superfusion system containing 15 ml of aerated (95% O_2/5% CO_2) Krebs solution maintained at 37°C. Muscle twitch was evoked by stimulating the phrenic nerve supra-maximally (0.2 Hz square waves of 0.1 ms duration and 1.5 V amplitude); muscle tension was measured using a force/displacement transducer attached to a polygraph [9]. Paralysis time was defined as that taken to give a 90% reduction of the initial nerve-evoked muscle tension [14].

3. RESULTS

The liposomes prepared in this study were required to encapsulate BoNT or its chains, and to be capable of fusing with the pre-synaptic membrane. To minimize the complication of toxin chains associating with the lipids rather than being entrapped inside vesicles, negatively-charged lipids were used for liposome preparation; this excludes electrostatic interaction of toxin or its chains (BoNT p$I \approx 6.3$) [11] with the liposomal lipids. However, the HC of BoNT is known to interact hydrophobically with liposomes at neutral pH [16]. Accordingly, when HC or BoNT was mixed with the lipids after sonication and the mixtures separated chromatographically (Fig. 1A,C) each associated with the liposomes, though to a limited extent (<5% of the total protein). Consistent with the lower hydrophobicity of the LC, its addition to

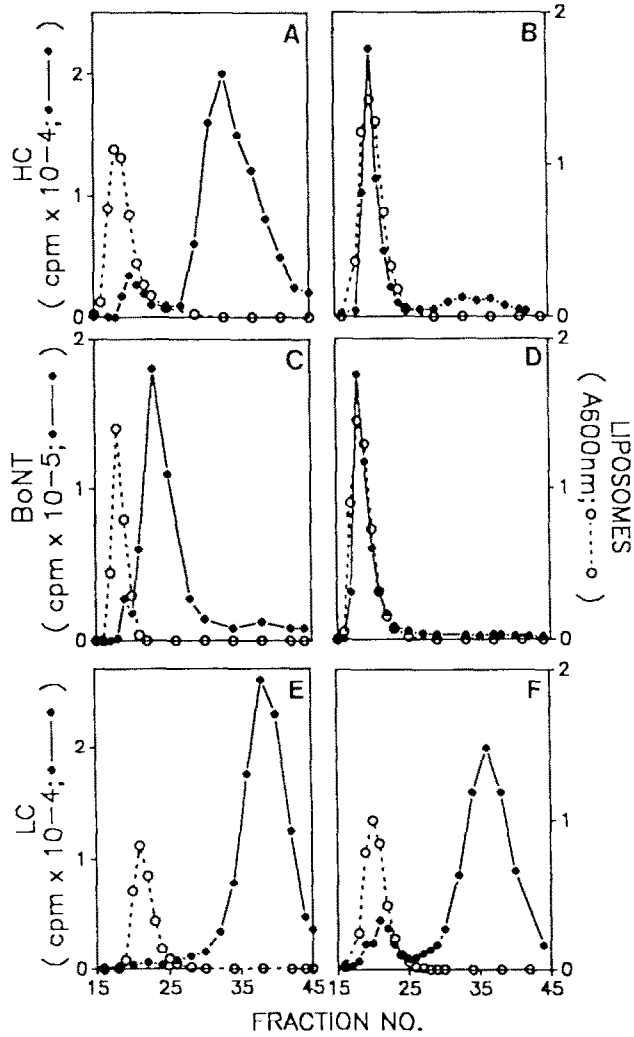

Fig. 1. Gel filtration of BoNT and its chains encapsulated in or associated with liposomes. Liposomes were prepared as described in section 2.4. The proteins together with radioiodinated tracer/liposome mixtures were loaded onto a Sephacryl S200 HR column and eluted at a flow rate of 1 ml/min and 1.5 ml fractions collected. The liposome and protein peaks were detected by determining the absorbance at 600 nm and radioactivity of each fraction. (A,C,E) Elution of preformed liposomes that were incubated for 30 min at 4°C with HC (A), BoNT (C) or LC (E) prior to application to the column. (B,D,F) Elution of liposomes containing HC (B), BoNT (D) or LC (F) prepared by sonication of the lipids in the presence of BoNT or its chains.

previously sonicated lipids resulted in negligible association with the liposome peak (Fig. 1E). At 37°C, the liposomes were fluid and did deliver their contents into both nerve and muscle; this was established by measurement of uptake of an inert radioactive tracer into endplate-enriched and non-endplate-containing areas of diaphragm (data not shown). Importantly such fusion of liposomes, devoid of toxin but containing the buffer used in the twitch experiments, failed to alter nerve-evoked muscle tension of diaphragm preparations (Fig. 2).

The level of encapsulation of HC into liposomes (Fig. 1B) was 70–80% of the total HC added (≈375 nM). When these liposomes were bath-applied to the medium superfusing the diaphragm, at a final concentration up to ≈75 nM, no detectable change in twitch tension resulted (Fig. 2); this is in accordance with the low toxicity of the HC preparations in mice (≈10^3 LD_{50}/mg; ≈10^5-fold less toxic than BoNT). In the case of the LC, 5–10% of that added (≈750 nM) to the lipid suspension became incorporated into liposomes (Fig. 1F). Notably, this liposome-entrapped LC decreased nerve-evoked muscle twitch tension (Fig. 2). In two separate experiments, ≈15 and ≈20 nM encapsulated LC (final concentration in the bath) gave 90% paralysis after ≈110 min with a lag time of ≈40 min. A third liposome preparation containing a lower concentration of LC

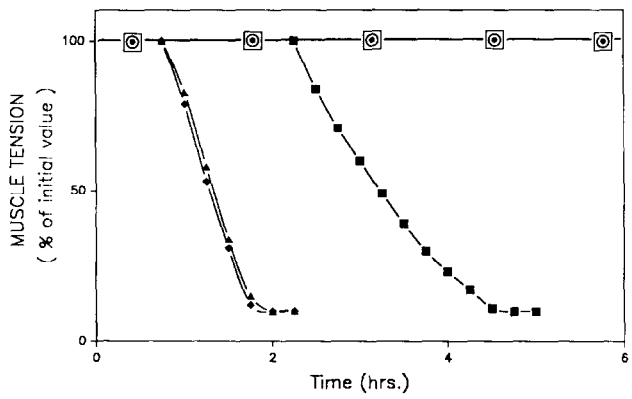

Fig. 2. Effect of control liposomes and those containing BoNT, HC or LC on neuromuscular transmission. Liposomes were dialysed into aerated Krebs solution and bath applied to mouse phrenic nerve-hemidiaphragm preparations which were superfused at 37°C and stimulated supra-maximally. Nerve-evoked muscle tension, expressed as a % of the initial value was measured against time. Control liposomes, lacking protein (•) failed to produce any change, as did liposomes entrapping HC (○) at a final concentration in the bath of up to ≈75 nM. Liposomes encapsulating LC at final concentrations of ≈20 nM (◆), ≈15 nM (▲) and ≈ 9 nM (■) decrease muscle tension. A fourth preparation of liposome-entrapped LC also gave the expected intoxication (data not shown). Liposome-entrapped BoNT at a final bath concentration of ≈0.2 pM (□), representing the maximum amount of contaminating BoNT that could have been present in the LC liposomes (see section 3), produced no change. The results shown for the control, HC and BoNT liposomes were each repeated twice; the data presented for the LC liposomes were obtained with different chain preparations.

(≈9 nM in the bath) elicited a decrease in nerve-evoked muscle tension with longer lag (≈140 min) and paralysis (≈280 min) times. Variation in the preparation of liposomes and the consequential differences in the extent of fusion probably underlies the disproportionate lower potency of the latter sample. It appears that a pre-synaptic blockade occurred because addition to the bath of 4-aminopyridine (4-AP; 0.2 mM) after paralysis by the LC liposomes caused a near-complete, though temporary, recovery of muscle-tension. 4-AP increases neuronal activity by increasing Ca^{2+} influx due to blockade of voltage-activated K^+ channels [15].

Furthermore, the neuroparalysis observed seems to be attributable solely to LC because no contaminants were revealed by SDS-PAGE and silver staining of the preparations used. Also, mouse bioassay indicated toxicities for LC of 10^3–10^4 LD_{50}/mg (at least 10^4-fold less toxic than the native toxin). To exclude the possibility that the toxicity seen with LC was due to any contaminating native toxin being encapsulated with the LC, liposomes were prepared with the maximum amount of BoNT that could have been present in the LC samples (calculated from the mouse toxicity values as 0.01%, *assuming* LC alone is non-toxic). The incorporation of 0.8 pM BoNT (Fig. 1D) into liposomes was again, like the HC, much higher (94–98%) than that of LC. No change in muscle tension was observed after addition of these liposomes (≈0.2 pM BoNT in the bath) to the nerve-diaphragm preparation (Fig. 2). Therefore, this study has shown that the LC, without the HC, once delivered intracellularly does inhibit ACh release from mammalian motor nerves.

4. DISCUSSION

The ability of liposomes to transport drugs and macromolecules across membrane barriers [17] has been utilized herein to deliver the HC or LC of BoNT into the cytoplasm of motor nerve terminals. Bypassing binding and internalisation in this manner allowed an assessment to be made of the chains' abilities to inhibit ACh release. The observed toxicity of liposomes containing LC establishes, for the first time, that it alone is capable of blocking synaptic transmission at mammalian nerve-muscle endplates, any contribution from contaminating native toxin having been eliminated. Also, these results reaffirm that the LC acts intra-cellularly because bath-application to the tissue of high concentrations (30 nM) of LC in the absence of liposomes failed to affect ACh release [9]. These findings accord with observations made on chromaffin [5,6,18] and PC-12 cells [7] using LC of BoNT or tetanus toxin. Moreover, the nature of the LC-induced pre-synaptic blockade resembles that of BoNT [15] because the neuroparalytic effects of both can be reversed temporarily by 4-AP. It is not readily possible to determine the absolute potency of liposome-

delivered LC due to the disproportionately large surface area of non-neuronal membrane compared to the minute amount of that from the pre-synaptic nerve terminal with which the liposomes could fuse.

Curiously, both chains of BoNT are required intracellularly for the inhibition of quantal release of ACh from *Aplysia* neurons [4]. This is suggestive of a secondary role for HC, particularly because LC alone of tetanus toxin is active in the same invertebrate neurons [10]. The success reported here in delivering LC into motor nerve endings could lead to a novel treatment for botulism. This would entail using non-toxic derivatives of BoNT to target liposomes with entrapped neutralising antibodies via interaction with ecto-acceptors unique to cholinergic nerve membranes [3].

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