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The major long-term goal of this contract is to develop a novel and effective treatment for human botulism by producing a drug, capable of neutralising the intracellular neuroparalytical action of botulinum neurotoxin (BoNT), and delivering this inside cholinergic neurons via a innocuous transporter form of the toxins. Towards this end, our multi-disciplinary research has demonstrated that BoNTs target to murine motor nerve endings by interaction with distinct ecto-acceptors uniquely located thereon; this 'productive' binding requires a conformation of the toxin's heavy chain (HC) that is maintained by its 'natural' association with the light chain (LC), even in the absence of the inter-chain disulphide. The subsequent acceptor-mediated uptake step could be monitored by high-resolution electron-microscopy of motor endplates labelled with BoNT-gold-conjugate, and from measurement of the inhibition of transmitter release produced by the resultant internalised LC. Importantly, experiments with modified forms of BoNT and its constituent chains revealed that the inter-chain S-S bond or its half-cysteines are essential for neuronal membrane translocation (..cont over)

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whereas neither the latter nor any of the toxin's free cysteines are necessary for intracellular blockade of transmitter release; in fact, alkylation of LC did not alter its ability to block acetylcholine release when administered intra-neuronally. However, a monoclonal antibody found to be reactive with a peptide sequence from near the N-terminus of LC neutralised and, actually, reversed the toxin's inhibition of neurotransmission; clearly, this domain is essential for neurotoxicity, a finding reaffirmed by site-directed mutagenesis. Thus, results to date dictate that the desired non-toxic transporter can be readily constructed by routine reconstitution (involving S-S bridging) of the BoNT HC with LC truncated at its N-terminus, a feasible task now being undertaken that should yield a vehicle capable of targetting to the nerve ending acceptors and translocating into the cholinergic neurons inactive LC, eventually with a covalently-attached BoNT-neutralising drug. Moreover, one form of the latter has already been produced i.e. anti-LC monoclonal antibodies with the demonstrated ability to counteract the effects of internalised BoNT.

Exciting progress has been afforded with the other essential facet of the project - the toxins' molecular action and the fundamental process of exocytosis - by the reported Zn^{2+}-dependent proteolytic cleavage by BoNT/B or tetanus toxin (TeTX) of synaptobrevin (Sbr) in brain synaptic vesicles. However, classical inhibitors of metalloproteases and ASQFETS, a hexapeptide that spans the cleavage site, were found to be poor inhibitors of Sbr breakdown by TeTX and virtually ineffective against BoNT/B. On the other hand, larger polypeptides corresponding to segments of Sbr sequence appear to interact much more strongly with these toxins and, thus, there is a growing prospect of devising a potent toxin inhibitor that could also be targetted via the cholinergic transporter. Although BoNT/A does not cleave Sbr, metal chelators and inhibitors of neutral endoproteases antagonise its action, and that of type B and TeTX, at the neuromuscular junction provided they are liposomally-delivered inside the nerve terminals. Apparently, all these *Clostridial* toxins act through their specific protease activities which are clearly distinct for BoNT/A and the others, though subtle differences were also revealed between BoNT/B and TeTX. The importance of Sbr in exocytosis, inferred from it being a target for some of these toxins, may relate to its possible interaction with microtubules because disassembly of the latter was demonstrated to reduce the efficacy of BoNT/B, E, F and TeTX (but contrastingly not BoNT/A). Unlike exocrine cells whose secretion process is BoNT/A resistant, use of permeabilised chromaffin cells is proving fruitful in allowing the functional site of action of BoNT/A and TeTX on exocytosis to be pinpointed; although both block the ATP-dependent but not the energy-independent component of secretion, they appear to act at distinct loci. Undoubtedly, continued application of these toxins as specific probes, particularly when used in conjunction with available antibodies to Sbr and other presynaptic proteins deemed to be involved in exocytosis, will yield mechanistic insights into the fundamental process; in turn, this should facilitate advances in development of a further generation of agents effective against botulism.

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

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ABBREVIATIONS: BoNT/type, botulinum neurotoxin types; HC and LC, heavy and light chains; TeTX, tetanus toxin; Red-A-BoNT/A, reduced-alkylated-BoNT/A; A-BoNT/A, non-reduced alkylated BoNT/A; A-LC, alkylated-LC; Ren-A-HC, renatured alkylated HC; SCVs, synaptic clear vesicles; LDCVs, large dense-core vesicles; ACh, acetylcholine; synaptobrevin, Sbr; KR, Krebs Ringer buffer; MKR, modified KR; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; DTT, dithiothreitol; ASW, artificial seawater; KGEP, buffer used with permeabilised chromaffin cells; DTPA, diethyltriaminepentaacetic anhydride; TPEN, tetrakis-(2-pyridylmethyl) ethylenediamine anhydride; NA, noradrenaline; mAb, monoclonal antibody; PTL, 1,10, phenanthroline; TBS, Tris-buffered saline; EM, electron microscopy; 4-AP, 4-aminopyridine.

SECTION 1. GENERAL INTRODUCTION

The long-term aim of this research program is to design a novel and effective treatment for human botulism, a usually fatal neuroparalytic condition caused by several structurally-related forms of botulinum neurotoxin (BoNT), from different species of *Clostridium botulinum*. All BoNT types (A-G), large proteins consisting of a disulphide-linked heavy (HC; M_r ~ 100kD) and light (LC; M_r ~ 50kD) chain, specifically and irreversibly block the release of acetylcholine (ACh) by targetting to motor nerve endings, via interaction with membrane ecto-acceptors uniquely located thereon, followed by efficient uptake and eventual inactivation of an intracellular and ubiquitous component that is essential for the release of all neurotransmitters (reviewed by **Dolly, 1992**). Our experimental strategy is to prepare an innocuous derivative of BoNT/A that would retain its unique abilities to bind to cholinergic presynaptic ecto-acceptors and become internalised, thereby serving as a membrane transporter to which BoNT-neutralising agents could be attached for delivery into the cholinergic nerve terminals. Thus, this project entails three main facets, as detailed in the original proposal, all of which have been investigated in the first half of the contract, focussing in separate sections on:

1. Deciphering the structural features of BoNT that are essential for ecto-acceptor binding, internalisation and intracellular action which will be paramount in the production of a cholinergic transporting vehicle.
2. Determining whether the various BoNT types have overlapping or distinct neuronal acceptors at mammalian endplates, information relevant to their exploitation for clinical purposes.
3. Elucidating the molecular mechanism of action of BoNT with a view to the future development of intracellularly-acting antagonists, suitable for conjugation to the BoNT transporter and with potential for the therapy of botulism.

Although ^{125}I-labelled BoNT/A and B have been shown to bind saturably (Dolly *et al.*, 1984) and exclusively (Black and Dolly, 1987) to cholinergic nerves and to be consequently internalised by an energy- and temperature-dependent process (Black and Dolly, 1986a, b), the limited resolution of the electron-microscopy (EM) autoradiography used precluded morphological evidence being obtained for acceptor-mediated endocytosis of BoNT. Hence, the development of a suitable colloidal-gold BoNT-conjugate is described here that allows neuronal trafficking to be followed, for the first time, by high resolution EM. In addition to elucidating the toxins' internalisation mechanism, this methodology will be essential for future monitoring of the binding, uptake and membrane translocation of a BoNT- based transporter. In order to investigate the 'productive' binding of toxin underlying uptake at the neuromuscular junction, a reduced / alkylated derivative (Red-A-BoNT/A) was used because it recognises the ecto-acceptors whilst a lack of toxicity permits its use as a functional antagonist (**Dolly *et al.*, 1992**). Advantageously, the 'active' binding conformation of the HC is maintained in the derivatised form, in contrast to renatured free HC (exposed to 2M urea during isolation) that exhibits low affinity for these mammalian acceptors (Bandyopadhyay *et al.*, 1987 ; Black and Dolly, 1986a). Furthermore, Red-A-BoNT/A was exploited in competition experiments to distinguish binding sites in this tissue preparation for the various *Clostridial* neurotoxins and, by use in conjunction with alkylated HC and LC, revealed that internalisation requires the toxin's inter-chain disulphide or its half-cystines. Finally, studies on the intra-neuronal activities of Red-A-BoNT/A or its alkylated LC (A-LC) showed that none of the thiols are essential for the intracellular blockade by LC of transmitter release. Thus, future efforts to inactivate LC for creation of a non-toxic transporter will focus on deletion or modification of its N-terminus because a monoclonal antibody (mAb) raised against LC of BoNT/A and reactive with a peptide sequence therefrom, was found to neutralise its intra-neuronal action, consistent with residues in this region being deemed essential for the latter, from results of molecular genetic studies (Niemann, 1991). In terms of obtaining a BoNT-neutralising agent for targetting purposes, it is important that this mAb and one other raised against LC of BoNT/A were found to be able to arrest and reverse (albeit temporarily) the BoNT/A-induced blockade of ACh release from *Aplysia* neurons, when administered intra-neuronally.

Investigations towards elucidating the molecular basis of the toxins' unique specificity in blocking intracellularly Ca^{2+}-dependent exocytosis continue to be of prime importance, both in terms of understanding this fundamental process and for developing drugs capable of arresting and / or reversing the intoxication. Based on the remarkable potency of BoNT, we and others had postulated an enzymic

mechanism (**Dolly *et al.*, 1987, 1990, 1992**). However, no convincing evidence could be obtained here for involvement of ADP-ribosyl-transferase activity (**Ashton *et al.*, 1988a, 1990**) or effects on phosphorylation systems in isolated central nerve terminals or synaptic clear vesicles (SCVs) (Dolly *et al.*, 1987; Ashton *et al.*, 1988b). Increased phosphorylation of a synaptic vesicle protein can be induced by BoNT/B under certain conditions but the inconsistency of the change casts doubts on its relevance. Likewise, it has proved impractical to confirm the ability of BoNT and tetanus toxin (TeTX) to enhance transglutaminase activity as reported recently for TeTx (Facchiano *et al.*, 1993); perhaps, our highly purified toxins lack such activity.

As we have suspected for some time that BoNT may exert a proteolytic action (USAMRIID application in 1988) on a synaptic vesicle protein, it was reassuring to read the recent evidence (Schiavo *et al.*, 1992 a, b) for TeTX and BoNT/B (but not A) being Zn^{2+} endoproteases that cleave synaptobrevin (Sbr) or VAMP II, a vesicle-associated membrane protein characteristic of SCVs (Trimble *et al.*, 1988). Although the sequence of all *Clostridial* neurotoxins possess the Zn^{2+}-binding motif (Kurazono *et al.*, 1992) and substitution with proline of the histidines concerned with co-ordinating the Zn^{2+} abolishes the activity of TeTX in *Aplysia* neurons (Niemann, 1991), for the proposal of this contract it was imperative to demonstrate that the same mechanism applied to mammalian cholinergic nerves. Towards this end, the effects of metal chelators, addition of excess Zn^{2+} or classical inhibitors of neutral metalloproteases on the neuromuscular blocking action of BoNT/A, B and TeTX were evaluated. It was pa ticularly pertinent to examine BoNT/A because of its inability to cleave Sbr despite having a Zn^{2+} motif, and in view of the well documented differences in its action from the other *Clostridial* toxins at central (**Ashton and Dolly, 1991; Ashton *et al.*, 1993**) and peripheral (Gansel *et al.*, 1987) nerve endings. For this facet, use of our established liposomal fusion system (**de Paiva and Dolly, 1990**) for delivery of the protease inhibitors into motor nerve terminals proved crucial.

Other reasons for actively pursuing this topic included the difficulties experienced here and elsewhere in reproducing the reported antagonism by captopril of TeTX- or BoNT/B-induced Sbr cleavage (Schiavo *et al.*, 1992a), or by using phosphoramidon and a Sbr-derived peptide known to diminish the action of TeTx inside *Aplysia* neurons (Schiavo *et al.*, 1992b). Removal of endogenous proteases by salt-washing SCVs was essential to diminish autolysis of Sbr in the absence of added toxin and to allow detection of the breakdown products produced by the toxins. Interestingly, ASQFETS, the peptide whose sequence spans the cleavage site of Sbr (Schiavo *et al.*, 1992a), and captopril, were shown to reduce Sbr proteolysis by TeTX but not BoNT/B. This revealing observation could prove invaluable in the design of toxin-neutralising drugs based on potent inhibitors of their protease action, for delivery with a transporter; it also highlights the importance of other subtle differences that are reported here in the actions of the various BoNT types. Whilst all the toxins were found to block release of transmitters from SCVs and large dense-core vesicles (LDCVs), that occurs at active zone and non-active zone regions of synaptosomal membrane (reviewed by McMahon and Nicholls, 1991), significant dissimilarities were noted in the level of reversal induced by a Ca^{2+} ionophore of active zone release after inhibition with different toxin types. Yet, our results show that all these toxins with the exception of BoNT/A (**Ashton and Dolly, 1991**) need intact microtubules in order to exhibit their maximal level of inhibition of synaptosomal transmitter release.

Lastly, the importance of Sbr in exocytosis, implied from its fragmentation by BoNT/B and TeTX, needs to be related to a precise role in the multi-step process. Thus, permeabilised cell systems have been employed in attempts to pinpoint the functional implications of the toxins' proteolytic actions. The observed BoNT/A- insensitivity of amylase secretion from pancreatic acinar cells suggests that its target is not a universal component of exocytosis, albeit essential in neurons and endocrine cells. Furthermore, an interesting difference was noted between BoNT/A and TeTX in their reduction of ATP-dependent catecholamine secretion from permeabilised chromaffin cells.

SECTION 2. DECIPHERING ESSENTIAL DESIGN FEATURES OF A BoNT-BASED MEMBRANE TRANSPORTER THAT TARGETS TO MAMMALIAN CHOLINERGIC NERVE ENDINGS

METHODS

(i) Monitoring of neuronal targetting and trafficking of BoNT.

Conjugation of toxin to colloidal gold particles - A commercial preparation (BioCell) of 2 nm gold particles was boiled for 5 min to disperse the colloid after storage and adjusted to pH 6.9, slightly below the isolelectric point of BoNT/A. To determine the optimum ratio for protein to gold colloid for conjugation, increasing amounts of toxin (0-65μg) were added to 1 ml of the colloidal gold and incubated for 10 min before stabilising with bovine serum albumin [BSA; 1% (w/v) final conc.]; complex formation was assayed using anti-BoNT/A LC antibodies. A diluted series of the rabbit antibody (**Cenci Di Bello *et al*, 1993**) was blotted onto nitrocellulose strips, air-dried for 30 min and then soaked in 5% BSA in phosphate buffered saline for 30 min at 37°C. After incubation with the different gold-labelled toxin conjugates for 4 h at room temperature, the strips were washed thoroughly in de-ionised water and incubated with the BioCell silver enhancing kit, to reveal the gold label as brown-black spots before terminating the reaction by washing the samples in tap water.

Tissue labelling - Small pieces of mouse phrenic nerve hemi-diaphragm were incubated with 1.15 ml conjugate in 5 ml of Krebs Ringer buffer (KR; 10 nM with respect to toxin content) at 22°C containing 0.2% (w/v) BSA and continuously gassed with 95%CO_2/5%O_2. Control samples were treated as before except that 100-fold excess of unlabelled BoNT/A was included in the incubation buffer. Tissue was fixed and processed for microscopy as described below.

Light microscopy - Cryostat sections (10 μm) of hemi-diaphragm labelled with the conjugate were cut on a microtome (Reichert-Jung) and thaw-mounted on gelatin-coated microscope slides. The sections were silver enhanced for 10 min at room temperature, washed with de-ionised water, then stained lightly for acetylcholinesterase to allow identification of endplates (Karnovsky and Roots, 1964), washed again and allowed to air dry. Slides were mounted with 90% glycerol (aqueous) and viewed in an Olympus light microscope.

Electron microscopy (EM)- After extensive washing with ice-cold buffer, the region of the diaphragm containing the phrenic nerve and rich in endplates was carefully dissected and cut into small strips (0.5 mm x 1 mm, shortest dimension parallel with the phrenic nerve). The strips were stained with 1% osmium tetroxide (aqueous) for 1 h at 4°C, dehydrated in ethanol and embedded in LR White resin. Ultrathin sections of pale gold interference colour were cut from the labelled tissue using an ultramicrotome (LKB Instruments) and collected on formvar-coated nickel grids. Sections were silver-enhanced for 10 min, washed with de-ionised water, stained with 1% uranyl acetate for 15 min and viewed in a Hitachi 600 EM. For assessment of the silver-enhancement protocol and comparison of labelling of endplates under different conditions, photographs were taken at a magnification of x 10,000 - 30,000. Measurements of grain size and distance from membranes were made directly from the negatives using a Mitotoyotu Comparator.

(ii) Preparation of Red-A-BoNT/A, alkylated BoNT/A and its renatured alkylated chains.

Highly purified BoNT/A (100 μg) isolated by an established procedure (Tse *et al*, 1982) and the reduced alkylated toxin (Red-A-BoNT/A) prepared by incubation with 50 mM dithiothreitol (DTT) for 60 min at 37°C and subsequently with 250 mM iodoacetamide for 30 min at 4°C in the dark, as detailed in **de Paiva *et al*. (1993).** BoNT/A was also alkylated, without prior reduction, with 150 mM iodoacetamide for 30 min at 4°C in the dark (to produce A-BoNT/A). Each preparation was immediately dialysed into KR prior to their application to mouse phrenic nerve-hemi-diaphragms, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, evaluation of the free thiol content (see below) and intra-peritoneal injection into mice for the determination of LD_{50}/mg, as described previously (Maisey *et al*, 1988). For the *Aplysia* experiments, Red-A-BoNT/A and A-BoNT/A were dialysed into artificial sea water (ASW). The modified toxin samples were subsequently centrifuged

(5000g, 5 min) to remove any insoluble material and the concentration of protein remaining in solution quantified, using the Bradford's assay (Bradford, 1976) with BSA as standard.

The chains of BoNT/A were separated by DTT reduction and denaturation with urea, followed by isolation using a routine chromatographic procedure (Maisey *et al.*, 1988); their purity was ascertained by SDS-PAGE and silver staining of the gels. Approximately 300 µg each of HC and LC were renatured upon removal of the urea and DTT by dialysis into Tris/NaCl buffer, which was thoroughly oxygenated prior to use. After dialysing for 72 h at 4°C with five changes of the aerated buffer, the chains were alkylated with 150 mM iodoacetamide for 30 min at 4°C in the dark; the resultant renatured, alkylated (Ren-A-H) and alkylated L (A-L) chains were dialysed into ASW before application to *Aplysia* neurons. Procedures are detailed in **de Paiva and Dolly (1993)**.

(iii) Characterisation of the above derivatives of BoNT and its chains.

Determination of the free sulphydryl content of the native and modified BoNT, HC and LC samples was carried out using a modification of the Ellman's reaction described previously (Ellman, 1959; Schiavo *et al.*, 1990). Subsequent to the dialysis of BoNT/A and its derivatives into KR, and the modified isolated chains into ASW, a 100 µg sample of each was precipitated with trichloroacetic acid [10% (w/v) final conc.) for 15 min, sedimented at 10,000g for 8 min and washed twice with 6% of the acid and once with de-aerated de-ionised water; all solutions were kept ice-cold. The protein was resolubilized in 0.8 ml of 50 mM Tris, 1 mM EDTA and 2% SDS, pH 8.0 by boiling for 20 min, thoroughly vortexing and leaving overnight at room temperature. Any bubbles were removed by gentle centrifugation at 2,000g for 2 min before the protein concentration was determined spectrophotometrically at 280 nm using an absorbance of 1.87 established experimentally to correspond to ~1 mg/ml SDS-denatured BoNT/A, and the equation $0.8\ (A_{235}-A_{280})/2.51 = 1$ mg/ml for the chains, as documented elsewhere (Bandyopadhay *et al.*, 1987; Whitaker and Granum, 1980). Thiol contents were assayed by measuring the absorbance at 412 nm (ε=14150 $M^{-1}cm^{-1}$) exactly 10 min after the addition of 30 µl of 5mg/ml stock dithionitrobenzene; 30µl of the latter in 0.8 ml solubilisation buffer was used as the reference blank. Modified toxin samples and the derivatised chains were prepared for SDS-PAGE (Laemlli, 1970) in 10% (w/v) acrylamide gels by boiling in sample buffer in the presence or absence of 5% β-mercaptoethanol.

(iv) Measurement of effects of toxin samples on transmitter release.

Action of derivatives of BoNT/A and its chains on transmission at cholinergic neurons of Aplysia - Intra-cellular recordings were performed on characterised cholinergic synapses of the buccal ganglion of *Aplysia californica* (Poulain *et al.*, 1988). The ganglion was pinned to the bottom of a 1 ml chamber and superfused (10ml/h) with ASW. The pre- and post-synaptic soma were impaled with two micro-pipettes filled with 3 M KCl (1-10MΩ). ACh release was evoked by a threshold pre-synaptic depolarisation and the ensuing response was recorded in the voltage-clamped, post-synaptic neuron as a current change and expressed as a membrane conductance (nS). The temperature of the superfusing medium was thermostatically controlled at 22-24°C except during bath-application of samples for competition experiments when it was lowered to 10°C, a condition sufficient to prevent BoNT internalization in this model (**Poulain *et al.*, 1989**). After the measurement of the control postsynaptic response, derivatised BoNT/A or its modified HC were added to the bath for 30 min followed by the application of 10 nM BoNT/A for 10 min. Subsequent to the removal of all unbound material by extensive washing for 1 h, the temperature was raised to 22°C and transmission monitored. Air-pressure intra-cellular administration of BoNT, its chains or their derivatives was accomplished as described elsewhere (Poulain *et al.*, 1988); identical micro-injection of control buffer solutions were shown to have no effect on transmitter release.

Assessment of the effect of modified toxin samples on mammalian neuromuscular transmission - Mouse phrenic nerve-hemi-diaphragms were excised from Balb C mice and bathed in a closed circulatory superfusion system containing 20 ml aerated KR (Maisey *et al.*, 1988). For the competition experiments, superfusion was halted and the tissues bathed in 10 ml of a modified KR solution (MKR, with the same composition as KR except with a Mg^{2+} and Ca^{2+} concentration of 5.0 mM and 0.5 mM, respectively) at 4°C to minimize toxin internalization (Black and Dolly, 1986b; Simpson, 1980). Following exposure of the phrenic nerve to modified toxin for 45 min, 0.2 nM BoNT/A was added to the bath. After a further

15 min, the nerve-diaphragms were washed thoroughly with MKR, then KR before the temperature of the bath was raised to 24°C. Superfusion was resumed and muscle twitch evoked by supra-maximal simulation of the phrenic nerve and measured using a force-displacement transducer, as described before (Maisey *et al.*, 1988). The neuroparalytic activities of A-BoNT/A and Red-A-BoNT/A relative to that of native BoNT/A were determined by their addition for 15 min to mouse diaphragms previously bathed in MKR only at 4°C for 45 min. The tissues were then washed and prepared for stimulation, as described for the competition experiments.

(v) Primary culture of bovine chromaffin cells and measurement of release of endogenous catecholamines from permeabilised tissue : assessment of the inhibitory effects of toxins and their derivatives.

Bovine adrenal glands were dissected and chromaffin cells prepared by protease perfusion, before being plated at 1 x 10^6 cells/well in 24 well plates in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 8 µM fluorodeoxyuridine, 50 µg/ml streptomycin (Dunn and Holz, 1983). Experiments were performed 3-8 days later and the cells were permeabilized with 20 µM digitonin in KGEP (representing in mM: potassium glutamate, 139; PIPES, 20; EGTA, 5; ATP, 2; $MgCl_2$, 2, pH 6.5) for 15 min in the presence or absence of 100 nM BoNT/A or Red-A-BoNT/A. The permeabilization buffer was subsequently replaced with KGEP or the latter containing Ca^{2+} buffered to a free concentration of 10 µM to stimulate secretion. After 15 min, the buffer was removed and assayed fluorimetrically to reveal the amount of Ca^{2+}-evoked catecholamine release (von Euler and Floding, 1955); basal release measured in the absence of Ca^{2+} was subtracted from the latter. The total cell catecholamine was evaluated by the same method subsequent to solubilizing the cells in the wells with Triton X-100 (1%, v/v).

RESULTS

(i) Targetting and trafficking of BoNT/A at motor nerve endings revealed by high-resolution colloidal-gold EM.

BoNT/A gold conjugate targets to murine muscle endplates via a saturable interaction with neuronal ecto-acceptors -In initial experiments, light microscopy was used to demonstrate the selective ability of BoNT/A gold-conjugate to reach cholinergic synapses via ecto-acceptors [see (ii) below], an essential feature of a cholinergic transporter. Binding of low concentrations of BoNT/A gold-conjugate (1 nM with respect to toxin content) was detected exclusively in endplate regions of mouse diaphragm (Fig. 1A) and this interaction was saturable, since binding could be completely inhibited by 100-fold excess of free toxin (Fig. 1B); such saturability was shown also at the EM level (see below). The specificity and saturability of conjugate binding was consistent with the appreciable level of retention of biological activity as measured in the mouse phrenic nerve-hemidiaphragm twitch preparation (>20%, data not shown). The attenuation of biological activity is most likely explained by a reduction in the ecto-acceptor affinity of the conjugate; it is not due to the presence of free BoNT/A since an optimal concentration of BoNT/A was used for conjugation with colloidal-gold that ensured it was all complexed to the gold particles. Reaction of the complex with anti-BoNT/A LC-antibody adsorbed on nitrocellulose strips was measured by detecting gold, by virtue of its association to toxin, bound to the antibody dots on the strips as brown / black spots after silver enhancing; the number of spots was maximal at 8 µg BoNT/A per ml colloidal gold (i.e. highest intensity of spots seen with the greatest dilution of antibody, representing the maximum amount of BoNT conjugate; Table 1). Higher ratios of toxin to colloid began to show some inhibiton of conjugate binding to antibody by free toxin indicated by a decline in the number of spots detected on the nitrocellulose strips (see Table 1). Further, in competition experiments, 10 nM free toxin gave a disproportionally higher level of competition of 10 nM conjugate binding to endplates than expected, i.e. labelling of the endplates was reduced by more than 50% (Fig. 1C, D), These results taken together show that BoNT/A can be conjugated to 2 nm colloidal gold preserving the specific and saturable binding to its acceptors at mammalian endplates, albeit with somewhat reduced affinity. Thus, this preparation can be used to follow the internalisation pathway determined by the ecto-acceptors.

Ultrastructural demonstration of BoNT/A-gold conjugate uptake and intraneuronal trafficking - This work highlights the feasibility of establishing, for the first time, an involvement of coated-pits, coated-vesicles and endocytotic compartments in BoNT trafficking by combining EM with colloidal gold-cytochemistry. To trap conjugate at various stages of endocytosis, a pathway implicated by the antagonism of BoNT poisoning by lysosomotropic agents (Simpson, 1989), a mouse diaphragm was incubated with conjugate for 30 min at room temperature; after this time, conjugate should be present in endocytotic compartments (Parton *et al.*, 1992). BoNT/A gold-conjugate was added to a final concentration of 10 nM (with respect to the toxin content) and the diaphragm was washed extensively with ice-cold KR buffer after incubation before processing for EM. Sections were silver enhanced in order to visualise the gold grains; this increased grain size linearly and a 2 nm particle could be grown rapidly and evenly to dimensions easily detected in electron micrographs. The location of grains was analysed; structures with which grains were associated were identified and grain size and distance to the nearest membrane were measured. At the nerve terminal a few grains were visualised at the cell surface and, excitingly, grains were found within coated vesicles implicating the acceptor-mediated pathway in BoNT/A neuronal uptake (Fig. 2A, B); also, this reaffirms that the site of interaction of conjugate with motor endplates is presynaptic. The interaction was specific and saturable since 100-fold excess of unconjugated BoNT/A inhibited conjugate binding (Fig. 2C), in agreement with the findings at the light microscope level. Coated-pits and coated-vesicles typically are associated with the earliest stages of acceptor-mediated endocytosis, although other endocytic routes do exist, for example caveolae-mediated potocytosis (Anderson *et al.*, 1992). Grains were frequently found in small smooth vesicles (Fig. 2D, E; see below), but it is currently not possible to distinguish between coated vesicles which have become uncoated prior to delivery of endocytosed material to endosomes, and synaptic vesicles which recycle from endocytotic compartments to the neuronal plasma membrane or even vesicles derived from smooth invaginations (caveolae) of the plasma membrane.

The majority of grains (75 out of 80) were found within membrane-bound structures which fell into 3 groups: uncoated vesicles, large vesicles and multi-vesicular bodies with mean sizes of 50, 165 and 400 nm, respectively (Fig. 2D-F, H). Some grains (23 out of 80) were centred on the membrane of the associated structure and for the remaining grains (52 out of 80) the mean distance to the nearest membrane was 12.2 nm ± 8.0 (S.D.) . Clearly, grains were associated with the membranes of labelled structures; the large vesicles and multi-vesicular bodies in which grains were located are typical endocytotic structures corresponding to early and late endosomes, respectively; the use of tracers such as α_2-macroglobulin-gold has revealed their distinct morphologies (Griffiths *et al.*, 1989). The presence of grains within vesicles contained in multi-vesicular bodies (Fig. 2E, F) revealed that some conjugate was destined for axonal transport and delivery to lysosomes (Parton *et al.*, 1992) and, indeed, the vesicles in multi-vesicular bodies are typically involved in routing acceptor-bound ligands to lysosomes. Interestingly, some grains were positioned directly over the membrane of small smooth vesicles (see above) and a few grains (5 out of 80) were not associated with membrane-bound organelles, but rather with filament-like structures in the cytoplasm (Fig. 2D, G, H). While it is tempting to speculate that these grains might represent toxin which has been translocated into the cytosol for subsequent interaction with its target, further work is needed on this point. These results can reflect that the conjugate reveals the intra-neuronal routing of toxin determined by interaction with its ecto-acceptor because it binds specifically and saturably to ecto-acceptors and remains closely associated with membrane-bound structures until translocated, i.e. the toxin is not released into endosomal lumen, which would occur if ligand-acceptor binding was disrupted by gold conjugation (Neutra *et al.*, 1985).

Studies are currently underway to determine the point of divergence of translocation of toxin into the cytosol and routes to other destinations by following the time course of uptake and appearance of grains at various locations in the nerve terminals. The proportions of grains destined for the various locations will also be determined. While it is likely that the conjugate follows the same pathway as unconjugated toxin, the decreased binding affinity compared to free toxin and unknown effects on ability to be translocated represent limitations which could be overcome with immunocytochemical methods using native toxin and anti-BoNT/A LC-antibody raised in this laboratory. Furthermore, this work is highly pertinent to the demonstration of targetting and trafficking of potential membrane transporters;

such an assay is required to determine whether the molecule can, indeed be, delivered to the cytosol the same way as the native toxin.

(ii) BoNT binding to productive ecto-acceptors at murine motor nerve endings requires a conformation of the HC maintained by its non-covalent association with LC.

The generation of Red-A-BoNT/A by the reduction of both disulphides prior to alkylation of the toxin, was ascertained by measuring the thiol content (Table 2). Mouse bioassay revealed that the toxicity of Red-A-BoNT/A was diminished to between 0.03 and 0.1% that of BoNT/A (Table 2); the residual toxicity may result from a remaining trace of non-reduced toxin. Consistently, it had a dramatically diminished activity compared to the native toxin; when bath-applied, 2.5 nM Red-A-BoNT/A was ineffective on neuromuscular transmission at the mouse nerve-diaphragm (Fig. 3A), while 10 nM was no effect on ACh release in the buccal ganglion of *Aplysia* (Fig. 3B). Binding competition experiments were undertaken with native BoNT/A and this freshly prepared derivative to ascertain whether this decrease in toxicity resulted from the loss of ability to bind to its productive ecto-acceptors. In the mammalian system, 2.5 nM Red-A-BoNT/A delayed the inhibition of ACh release due to 0.2 nM BoNT/A by ~3 h (Fig. 3C), while the action of 10 nM BoNT/A on *Aplysia* neurons (Fig.3D) was completely blocked for at least 2 h by a 10-fold molar excess of the modified toxin. Importantly, these experiments demonstrate that Red-A-BoNT/A, although very much less toxic than the native toxin, appears to have retained the ability to bind to its acceptors in both the models tested.

Notably, when the HC was employed similarly in a binding competition study at the neuromuscular junction, 30 nM HC was unable to delay the action of 0.3 nM BoNT/A (Fig. 3E). In contrast, 100 nM HC blocked the binding of 10 nM BoNT/A at cholinergic neurons of *Aplysia* (Fig. 3F). This confirms our published data (**Poulain *et al.*, 1989, 1993**) and reiterates the discrepancy in the requirements for binding at mammalian and invertebrate neurons; apparently, there is a more stringent structural requirement for toxin binding at neuromuscular junction than at *Aplysia* nerve terminals. It should be noted, however, that Bandyopadhay *et al.*, (1987) and Lomneth *et al.*, (1990) did observe an antagonism of the action of a high BoNT/A concentration (10 nM) by 1 μM HC at murine and avian muscle endplates. Although the basis for this apparent contradiction remains unknown, the relatively tight binding of Red-A-BoNT/A to mammalian neuronal ecto-acceptors is much more avid than that of the isolated HC. Thus, this non-toxic derivative of BoNT/A provides, for the first time, a tool suitable for the investigation of the productive binding of the toxin and, thereby, a possible means to discern the nature of these acceptors.

(iii) The inter-chain disulphide or half-cystines of BoNT are essential for the HC-mediated internalisation of LC at cholinergic neurons.

Despite Red-A-BoNT/A having a largely diminished neurotoxicity, it retained its ability to bind to the toxin's productive acceptors (see above); thus, its near complete loss of activity when applied extracellularly must be due either to an inability to undergo membrane translocation or to exert the intracellular inhibitory effects characteristic of BoNT/A. As revealed in the following sub-section (iv), Red-A-BoNT/A was indeed capable of blocking transmitter release when intra-neuronally micro-injected into neurons of the buccal ganglion from *Aplysia* or applied to digitonin-permeabilised bovine adrenal chromaffin cells. Moreover, the free sulphydryls were shown not to be required for any phase of the toxins action, as the alkylation of the non-reduced toxin did not alter its toxicity in mice, or inhibitory action when bath-applied to the mouse phrenic nerve hemi-diaphragm preparation or *Aplysia* neurons. Collectively, this indicates that the disulphides or the half cystines involved play an important role in the process by which the LC is translocated across the membrane into the cytosol. Due to the inability to selectivity reduces the inter-chain bond, as was found elsewhere with TeTX using thioredoxin and thioredoxin reductase from *E.coli* (Schiavo *et al.*, 1990), an alternative approach was adopted. For this purpose, homogeneous samples of Ren-A-H and A-LC (Fig. 4) were prepared and evidence for their reduction and alkylation was provided by SDS-PAGE and evaluation of their thiol contents. The electrophoretic mobility of Ren-A-HC, when run under non-reducing conditions, was detectably lower than that of the same derivative or HC following exposure to β-mercaptoethanol (Fig. 4). Additionally,

the sulfhydryl content of Ren-A-HC increased from 0.2 ± 0.1 to 2.7 ± 0.2 (Table 3) upon incubation of this derivative with DTT (100 mM), confirming that the disulfide within this derivative had reformed upon renaturation of the alkylated HC. This data also reaffirmed that all the other thiols were alkylated including, most pertinently, the residue involved in the formation of the disulfide bridge with the LC, cysteine 454. All thiols in A-LC were modified as demonstrated by the measured thiol content (Table 3). As shown above, the HC of BoNT/A is incapable of binding to the toxin's functional acceptors at the neuromuscular junction under the strict conditions used (Maisey *et al.*, 1988) but does bind to such sites in *Aplysia* neurons (**Poulain *et al.*, 1989**). For evaluating the ability of the Ren-A-HC to bind, the capability of HC to antagonize BoNT/A action in *Aplysia* (**Poulain *et al.*, 1989**) has been exploited. Under conditions designed to minimize toxin internalization (10°C), pre-incubation of the preparation with Ren-A-H (100 nM) prevented the inhibitory action of 10 nM BoNT/A after returning the temperature to 22°C (Fig. 5A). This confirmed that the modified HC has retained the ability to bind to acceptors on *Aplysia* neurons. As previously published (**Poulain *et al.*, 1988, 1989a, 1990**), a mixture of the LC and HC (or, at least, the LC plus the HC domain) of BoNT/A is required intra-cellularly to inhibit neurotransmitter release in this invertebrate system. In contrast with the results of previous experiments where extra-cellularly applied mixtures of the native HC and LC from BoNT/A inhibited ACh release from these neurons (Maisey *et al.*, 1988), Ren-A-HC (100 nM) bath-applied together with the unmodified LC (100 nM) failed to affect transmitter release (data not shown). This indicates that either the Ren-A-H or the LC, or both, failed to enter this neuron because both are required intra-cellularly for toxicity (see above). To investigate this, HC (10 nM, final concentration) was injected into the pre-synaptic neuron (to provide the HC domain required for the blockade of ACh release should the extra-cellularly-applied LC become internalized) prior to the bath-application of Ren-A-H (100 nM) and LC (100 nM) (Fig. 5C). As no decrease in neurotransmitter release was detected, it appears that the LC was not internalized by Ren-A-HC. Additionally, Ren-A-HC alone was shown to be unable to undergo internalization because pre-injection of LC followed by extra-cellular application of Ren-A-HC failed to inhibit ACh release (Fig. 5B), contrary to the inhibition which ensues following addition to the bath of unmodified HC and pre-injection of the LC (Poulain *et al.*, 1988). Since the bath-application of A-L (100 M) together with an equimolar amount of HC also failed to elicit any change in the evoked release of transmitter (Fig. 5D), it was concluded that the HC is unable to internalize A-LC; thus, modification of the cysteines within the LC is sufficient to disrupt the internalization process - as alkylation of those thiols not involved in the inter-molecular bond has no effect on toxin uptake (see below), it is apparent that modification of the thiol involved in this bond is involved in the toxin's action. Collectively, these results suggest that toxin uptake is prevented by the reduction of the inter-chain disulfide or the alkylation of either one of the participating cysteine residues. Importantly, these results highlight that in designing a cholinergic transmembrane transporter, the inter-chain disulphide and, thus, at least the C-terminal portion of the LC containing the cysteine involved in this bond must be retained.

(iv) Distinguishing structural features of BoNT or its LC that are essential or unnecessary for their intracellular action.

Having established that the inter-chain disulphide or the half-cystines are integral to the internalisation phase of this toxin, their requirement in its ultimate intracellular stage of paralysis were investigated. BoNT/A alkylated with iodoacetamide without prior reduction of its disulphides (A-BoNT/A) was as potent as the native toxin in the mouse bioassay (Table 3), in paralysing neuromuscular transmission (Fig. 6A) and in blocking ACh release in *Aplysia* (Fig. 6B). Despite the almost complete absence of activity from Red-A-BoNT/A when injected into mice or bath applied to mammalian or vertebrate neuronal preparations (see above), when intracellularly applied it was as efficacious as the unmodified toxin in permeabilised chromaffin cells and *Aplysia* (Fig. 6C, D). Correspondingly, micro-injection of Ren-A-HC (10 nM, final intra-neuronal conc.) together with an equal concentration of the modified LC or the same amount of native HC mixed with A-LC (10 nM, final conc.) both elicited a decrease in evoked transmitter release in the *Aplysia* neurons (Fig. 6E). This re-iterates that neither the toxin's inter-chain disulphide bond, half-cystines nor free thiols are required for its intracellular inhibition of transmitter release.

A successful BoNT-based transporter must encompass an ability to bind and cross the membrane in order to gain access to the cytosol where it must obviously have lost the ability to exert its neuroparalytic effects. The generation of Red-A-BoNT/A has shown that neither the disulphides nor thiols are required for high affinity toxin binding, but importantly, an intact disulphide is essential for membrane translocation and, thus, must be incorporated into a transport vehicle. As demonstrated here, the detoxification of the LC which contains the toxins' active site (**de Paiva and Dolly, 1990**) must involve a different approach from chemical modification of the half-cystines or thiols. The ability of the LC of BoNT/A to block ACh release in *Aplysia* is abolished following the deletion of 63 C-terminal or 10 N-terminal residues (Kurazono *et al.*, 1992). Importantly, the deletion of 62 C-terminal residues, including the cysteine (430) involved in the inter-chain disulphide, did not perturb it toxic activity in *Aplysia*. However, the loss of toxicity following the removal of the first 10 N-terminal residues, and the prevention plus reversal of the toxin's action using a mAb directed towards the N-terminal region [see section (v) below] may indicate a convenient means by which to prepare a non-toxic transporter targetted preferentially to peripheral cholinergic neurons. This could consist of the entire HC disulphide-linked to the LC, with at least 10 N-terminal residues missing. Alternatively, the LC may be inactivated by site directed mutagenesis of the glutamate-223, which probably plays a catalytic role in the toxins' active site (see Section 3) or by substitution, of at least two of three histidines which are essential for the co-ordination of the Zn^{2+} in the toxins active site (Schiavo *et al.*, 1992b).

(v) mAbs against the LC of BoNT/A that reverse the toxins intra-neuronal action: a prerequisite for their use in the development of a targetted antagonist.

In the final report of our previous contract, the generation of mAbs specific for the LC of BoNT/A was reported. More recently, a number of additional mAbs have been produced (**Cenci di Bello *et al.*, 1993**) using the same procedure and these have been extensively characterised. The seven established hybridoma cell lines secreted mAbs of IgG_1 and IgG_{2b} subclasses and reacted specifically with BoNT/A and LC, in the native and denatured states, without showing any cross-reactivity with types B, E, F or TeTX (Fig. 7). The pronounced reactivities of three mAbs towards refolded LC or intact toxin, observed in immuno-binding and precipitation assays, relative to that seen in Western blots imply a preference for conformational epitopes (Table 4). Though mAbs 4, 5 and 7 failed to neutralise the lethality of BoNT *in vivo*, pre-administration of mAb7 inside *Aplysia* neurons prevented the inhibition of transmitter release normally induced by subsequent extra-cellular administration of BoNT/A, as documented in the final report of the previous contract (Note mAb 7 was then termed G5). Notably, this mAb reacted with a synthetic peptide corresponding to amino acids 28-53 in the N-terminus of the LC, a highly conserved region in *Clostridial* neurotoxins reported to be essential for maintaining the chains tertiary structure (Kurazono *et al.*, 1992). mAb7 did not recognise a peptide corresponding to the first 16 residues of the N-terminal of BoNT LC (**Cenci di Bello *et al.*, 1993**); this was kindly provided by Dr. Jim Schmidt (USAMRIID). Most importantly, when mAbs 4 or 7 were micro-injected inside ganglionic neurons of *Aplysia*, each totally reversed, though transiently, the toxin's blockade of ACh release (Fig. 8). As described in section 4, an enzymic basis for the mode of BoNT/A action has been shown, although the target remains to be identified. The observed reversal of toxin action by mAb 4 and 7 has provided some insights into the properties of this intra-neuronal target. The fact that it is possible to reverse the intoxication process, even transiently, clearly indicates that the substrate for BoNT/A has either a high turnover or that it exists in a form which is not directly accessible for proteolysis; for example, in only certain pools of vesicles from the several that have been documented (Prado *et al.*, 1992). A BoNT-susceptible protein with a high turnover being implicated in the mechanism of neurotransmitter release is unlikely as treatment of *Aplysia* synapses with the protein synthesis inhibitor, cycloheximide, was without effect on neurotransmission (Mochida *et al.*,1990). In the context of the results presented herein, if recovery was due to a high turnover of the target, it should have been possible to restore neurotransmission independently of the time at which anti-LC mAbs were applied. But this was not the case, indicating that the total amount of target is exhaustable and that the so-called reservoir should be in a dynamic equilibrium with the pool susceptible to the toxin. The fact that the recovery was only transient cannot be interpreted to mean that the mAbs were unable to 'buffer' all the toxin and a sufficient fraction

of un-neutralised surplus BoNT was left to block release because, under some conditions, near complete recovery of neurotransmission was seen even when release had been reduced by BoNT to less than 70% of the control level. Hence, these findings suggest that BoNT, through its postulated proteolytic action (or whatever mechanism) inactivates a selected pool of vesicular membranes or species of cytosolic proteins involved in the exocytotic pathway that have a rapid intracellular redistribution. The inability of these mAbs to neutralise BoNT action *in vivo* illustrates a limitation of this system and shows that only by circumventing membrane barriers, thereby facilitating direct introduction into neurons, can an adequate assessment of selected mAbs' neutralising activities be made. Potentially, this may be achieved using a non-toxic transporter based on the neurotoxin as described in the above sub-section (iv).

SECTION 3. DEMONSTRATION OF DISTINCT 'PRODUCTIVE' NEURONAL ECTO-ACCEPTORS FOR SOME BoNT SEROTYPES AND TeTX THAT COULD BE EXPLOITED FOR TARGETTING AND CLINICAL APPLICATIONS

METHODS

(i) Binding competition studies with TeTX and various serotypes of BoNT at mammalian motor nerve endings.

Under the optimal conditions utilised in Section 2 to measure the interaction of BoNT and Red-A-BoNT/A with 'productive' toxin acceptors, 2.5 nM Red-A-BoNT/A was applied to the mouse phrenic nerve hemi-diaphragm. After 45 min at 4°C BoNT/B (0.4 nM), BoNT/E, F (0.2 nM) or TeTX (5.0 nM) were added and the tissue incubated for a further 15 min. The diaphragms were subsequently washed and the temperature raised to 24 or 37°C for the BoNT and TeTX incubations, respectively. Upon stimulation of the phrenic nerve, the ability of Red-A-BoNT/A to delay the onset of neuroparalysis induced by the BoNT serotypes or TeTX was assessed by comparison with their actions when applied for 15 min at 4°C to diaphragms, that had been maintained in MKR only at 4°C for 45 min [see Methods 2(iv)].

(ii) Measurement of neurotransmitter secretion from brain nerve terminals.

Rat cerebral cortical synaptosomes (P2 fraction) were prepared as outlined **(Ashton and Dolly, 1991)**; release of different neurotransmitters were measured after prior incubation of nerve terminals with radiolabelled transmitter/precursor. Various compounds were present throughout the experiment to prevent degradation of the radiolabelled compounds; pargyline and ascorbic acid with [^{3}H] noradrenaline (NA); aminooxyacetic acid with [^{3}H]GABA, and eserine or neostigmine sulphate with [^{3}H]choline. Prelabelling was carried out concurrently with intoxication of the synaptosomes because the toxins did not interfere with the former. At the appropriate times, the toxins and excess radioactivity were removed by several washing steps involving the centrifugation/resuspension of the synaptosomes. At this time Ca^{2+} was removed by the inclusion of EGTA in the first 2 washes; aliquots of these terminals were then stimulated with an 2elevated K^+ concentration, 4-aminopyridine or Ca^{2+} ionophore. Ca^{2+}-dependent evoked release was determined by measuring secretion in its presence or absence over a period of 5 min, prior to termination by centrifugation and supernatant removal (see Ashton and Dolly, 1988). For NA and GABA, the amount of radioactivity in the supernatant was quantified by scintillation counting and presumed to be intact transmitter; also, secretion was induced in Na^+-free medium (Na^+ replaced by choline chloride) in an attempt to reduce release occurring via the reversal of Na^+-dependent GABA uptake (Santos *et al.*, 1991). The transmitter remaining within the synaptosomes was determined after the solubilisation of pellets. Both choline and ACh present in the supernatant from cholinergic terminals were seperated by incubation with choline kinase and MgATP. The phosphocholine produced therein was seperated from ACh by organic extraction with sodium tetraphenylboron in heptanone; the former remains in the aqueous phase whilst the latter enters the organic solvent. The aqueous phase is removed and radioactivity determined whilst the ACh can be assessed after re-extraction from the organic layer with HCl. The total content of choline and

ACh inside the nerve terminal was determined in a similar manner after the pellets were extracted with acetone/formic acid and the amount of release was expressed as % of total content. Often in intoxication experiments, values were expressed relative to the release from the non-toxin treated control, which was set at a 100%.

RESULTS

(i) Red-A-BoNT/A - a functional antagonist shows that BoNT/B, E, F and TeTX do not significantly share acceptors with BoNT/A.

Establishing the existence of distinct ecto-acceptors targetting each toxin to motor nerve endings and their role in determining the efficacies of these neurotoxins has been relatively unsuccessful, until now, due to a lack of high-affinity pharmacological antagonists of the toxins' binding. The presence of distinct sites has been indicated by studies with peripheral nerve preparations using the HC from BoNT/A (Bandhyopadyay *et al.*, 1987), the isolated 50 kDa C-terminus fragment from TeTX (Simpson, 1984) and ^{125}I-labelled BoNT/A and B in peripheral (Black and Dolly, 1986a) and central (Williams *et al.*, 1983; Evans *et al.*, 1986; Wadsworth *et al.*, 1990) neurons preparations. However, due to the high molar excess of antagonist, over the native toxins, required for a reasonable hindrance of toxin binding, the relevance of these sites to the intoxication process has not been ascertained. In contrast, Red-A-BoNT/A, described in Section 2, inhibits the native toxin's functional binding at low concentrations, thereby allowing for the discrimination of the 'productive' binding sites of BoNT/A from those for types B, E, F and TeTX (**Ashton *et al.*, 1993**). The modified toxin did not delay the actions of 0.2 nM BoNT/F or TeTX (5.0 nM) while that of 0.4 nM BoNT/B and 0.2 nM type E was marginally diminished (Fig. 9). This establishes that BoNT/A does not share its pertinent physiological acceptors with BoNT/F or TeTX while a small degree of overlap may exist between those for BoNT/A and types B and E; the latter might have resulted from steric hindrance of toxin binding to separate but adjacent sites. The apparent presence of distinct acceptors has important clinical implications as they provide multiple targets for potential use in treatment of botulism and in the design of cholinergic transporters based on the these toxins.

(ii) Various BoNT types and TeTX display characteristic potencies in reducing release of different transmitters from rat cerebrocortical synaptosomes.

It is well established that much higher concentrations of BoNT/A are required to block ACh release from brain synaptosomes than those effective at peripheral motor nerves; in fact, central cholinergic nerve terminals show only marginally greater susceptibility than other transmitter types (Ashton and Dolly, 1988; **Ashton *et al.*, 1993**). Although inefficient uptake has been postulated for this low potency of BoNT/A (Black and Dolly 1987), type B is near-equipotent with A and F towards NA efflux (Fig. 10A) whilst being considerably more effective in blocking synaptosomal ACh release (Fig. 10B). More noteworthy is the remarkable ability of BoNT/E to abolish K^+-evoked, Ca^{2+}-dependent release of NA relative to types A, B, F or TeTX (Fig. 10A,C). Likewise, TeTX is much more potent at blocking the release of the inhibitory neurotransmitter, GABA, than catecholamine release (Fig. 10C). Although such a striking range of potencies could be attributable to one or more steps in the triphasic intoxication process, at least some of these effects must be due to the differences in ecto-acceptors. In this context, the central cholinergic acceptors for BoNT/B may be of similar affinity as those at peripheral motor nerve endings; if so, synaptosomal membranes would provide a good tissue source for much needed bicchemical investigations on this acceptor which are proving difficult with peripheral neuronal preparations.

SECTION 4. BoNT/A, B AND TeTX ARE Zn^{2+} ENDOPROTEASES : FUNCTIONAL IMPLICATIONS OF THE CLEAVAGE OF Sbr BY BoNT/B AND TeTX

METHODS

(i) Quantitation of Zn^{2+} content of BoNT/A.

All buffers and solutions were made with water that had been de-ionized and purified by reverse osmosis. Additonally, this water was treated with resin containing immobilized EDTA (Chelex) to remove adventitious metal ions. All plasticware employed was treated with 30 % nitric acid, followed by careful rinsing in metal-free water. Dialysis tubing was rendered devoid of metals by extensive washing in metal-free water at 85°C. Homogenous BoNT A (as assessed by silver stained SDS-PAGE gels) was dialyzed at a conc. of 2 mg/ml for 3 days against 4 changes of 25 mM Hepes pH 7.5. to remove adventitious metals. Samples were treated with 5 % nitric acid prior to metal analysis on a flame atomic absorption spectrometer; the matrix buffer and nitric acid contained no detectable Zn^{2+} when compared to Zn^{2+} standards.

(ii) Measurement of the effects of removal and addition of Zn^{2+} on the toxins' action at mouse motor endplates.

Although preparations of BoNT/A and B were homogeneous as judged by silver staining of the proteins after SDS-PAGE (not shown), a supplementary purification step was employed for those experiments investigating the protease nature of the toxins. Following the dialysis of the isolated BoNT/A or B samples into 50 mM sodium succinate, pH 5.5 (overnight at 4°C), 4.5 ml of the toxins were loaded via a 10 ml loading loop onto a FPLC 10/10 Mono-S column (column volume ~10 ml). Subsequent to washing with the same buffer (20 ml at 5 ml/min), a salt gradient (0 - 0.3 M NaCl over 20 min) was initiated. The eluted protein, collected in 1 ml fractions, was detected by measuring the absorbance at 280 nm; the peak protein fractions were pooled and dialysed immediately into a Hepes buffer (25 mM, pH 7.4) containing NaCl (150 mM). The purified BoNT/B, of which ~50% was of single chain species, was trypsinised to generate a homogeneous sample of di-chain toxin, as described before (Maisey *et al.*, 1988). Either neurotoxin (0.2-0.5 μM, final concentration) in the above Hepes / NaCl buffer were incubated with 10 mM phenanthroline (PTL) for 2 h at 37°C and subsequently dialysed into 150 mM Tris-HCl, pH 8.0; this latter buffer was prepared with high purity water (conductivity <0.07 $\mu S.cm^{-1}$) and all plastic plus glassware were soaked in EDTA (~5g.l^{-1}) and rinsed thoroughly in the de-ionised water. These samples were applied to the KR superfusing the mouse phrenic nerve hemi-diaphragms (50pM BoNT/A or 80 pM BoNT/B) and their neuroparalytic activities compared to those of the control untreated toxin samples. To minimise the free Zn^{2+} content in the physiological medium bathing the tissues, 50 μM diethyltriaminepentaacetic anhydride (DTPA) and 30 μM tetrakis-(2-pyridylmethyl) ethylenediamine anhydride (TPEN) were added to certain of the nerve muscle preparations prior (for 2 h, with stimulation of the phrenic nerve) and following the application of the toxins. Suitable control experiments revealed that these chelators did not perturb the normal nerve-evoked muscle tension. Moreover, PTL-treated and control BoNT/A and B, following their dialysis into the metal-free Tris-HCl, were stored for 2 h at 4°C with added Zn^{2+} acetate (50 μM or 30 mM, final conc.) before their application to the tissues bathed in KR without DTPA or TPEN.

(iii) Preparation of liposomes and lipofusion at mouse neuromuscular junction.

Liposomes containing phosphoramidon or captopril - Liposomes known to deliver their contents intra-neuronally to the pre-synapse of the mouse phrenic nerve-hemidiaphragm (**de Paiva and Dolly, 1990**) were employed to transport phosphoramidon and captopril to this site. Phosphatidyl choline, cholesterol and phosphatidyl serine were dried [(total 40 mg in a 7:2:1 (w/w ratio)] before mixing with a solution of phosphoramidon or captopril (10 ml of 200 μM) in a Hepes (10 mM, pH 7.4) and NaCl (118 mM) buffer. Liposomes were formed, as detailed before (**de Paiva and Dolly, 1990**), using a probe sonicator, but in contrast to this original protocol, the un-entrapped material was separated from the encapsulated inhibitors by centrifugation of the vesicles for 40 min at 229,000 g. The pellet was resuspended in 10ml

of the KR, warmed to 37°C, aerated with 95% O_2/5% CO_2 and 5 ml of this added to diaphragm preparations (total bath volume 10 ml).

Liposomes entrapping phosphoramidon - treated and untreated LC of BoNT/A - A sample of the LC of BoNT/A (~0.8mg) was dialysed into a Hepes (10 mM, pH 7.4) and NaCl (118 mM) buffer over 24 h at 4°C and divided into two equal halves. Prior to this division of the LC, a small quantity of [^{125}I]-labelled LC, prepared by radio-iodination of BoNT/A using the chloramine T method (Williams *et al*, 1983) and the subsequent isolation of the labelled LC, was added to act as a tracer during the manufacture of the liposomes and to estimate the quantity of LC ultimately added to the nerve-muscle preparations. Phosphoramidon (200 μM, final conc.) was added to the first LC sample prior to its encapsulation within liposomes; the remainder of the LC was entrapped without prior exposure to phosphoramidon. The unencapsulated material was separated from the liposomes using a Sephacryl S200 HR gel filtration column and the latter were added to the murine phrenic nerve-hemi-diaphragm preparations following their dialysis overnight (at 4°C) into the KR. The abilities of the phosphoramidon-treated and control liposome-entrapped LC to block neurotransmission were compared after ensuring that equal amounts of LC (10-15 nM final conc in the bath) were applied to each of the two tissue baths, by measuring the radioactivity present.

(iv) Preparation of Sbr-derived peptides.

For inhibitory studies on the toxins' protease activity, a peptide (ASQFETS) corresponding to the cleavage site of rat Sbr II (Schiavo *et al.*, 1992a) was synthesized on an automated apparatus (Applied Biosystems) by the F moc solid phase method. The peptide was >95% pure by reverse-phase HPLC and its primary sequenced was confirmed by Edman degradation and the detection of expected molecular ions by fast atom bombardment mass spectrometry. Another peptide, residues 55 to 70 of Sbr II, was also synthesized; this was 75 % pure by analytical HPLC and its main molecular ion corresponded to the correct sequence. It was used as an antigen to elicit an antibody response in rabbits.

(v) Generation of Sbr-specific antibodies using synthetic peptides as antigens.

Residues 55-70 of rat Sbr II were chosen as the antigen primarily because this sequence is very hydrophilic and likely to be surface orientated and antibody reactive. Additionally, this region is completely conserved between the two isoforms, and in all species studied. The peptide was coupled to keyhole limpet haemocyanin using the crosslinker, MBS, via an N-terminal cysteine residue added to the peptide for this purpose. Immunization and antibody screening were carried out using established methods. Peptide reactive antibodies were affinity purified using the peptide immobilised on amino-butyl Sepharose 4B (EDC method); immunoreactivity of the anti-peptide antibodies towards the parent Sbr was assessed using Western blotting.

(vi) Purification and salt treatment of synaptic vesicles.

SCVs were isolated by an adaption of the method of Huttner *et al. (1983)*

P2 preparation of rat cerebral cortical synaptosomes

↓

Osmotically shock in 5 mM hepes buffer (pH 7.4) and homogenize using 12 strokes of Potter type homogenizer. Leave on ice for 30 min with stirring

↓

Centrifuge the homogenate at 25,000 x g_{av} for 25 min

↓

Centrifuge the supernatant at 165,000 x g_{av} for 120 min

↓

Resuspend pellet in 6 ml 40 mM sucrose (buffered with 5 mM Hepes pH 7.4) and homogenise using 10 strokes of homogenizer. Mix homogenate further by forcing solution back and fourth through a 25-guage needle. This is then layered on top of a linear sucrose gradient comprising of 25 ml 800 mM buffered sucrose and 29 ml 50 mM buffered sucrose. Centrifuge at 65,000 x g_{av} for 300 min

↓

Layer relevant fractions (see Fig. 15) onto a Sephacryl S-1000 column pre-equilibrated in 0.3 M glycine 5 mM Hepes pH7.4. Collect the vesicle fraction by elution with the same buffer and pellet by centrifugation at 200,000 g_{av} for 30 min.

All buffers contained a cocktail of protease inhibitors (0.2 mM PMSF, 1mg/ml peptstatin A, with or without 1 mM EDTA). Purified SCVs vesicles were resuspended at ~1 mg/ml protein in 0.3 M glycine 0.005 M Hepes pH 7.4, and stored at -20°C. Less pure vesicles from the sucrose gradient could be employed in experiments provided that these were salt-washed; this entailed diluting the organelles in 0.3 M glycine 0.005 M Hepes pH 7.4, and adding solid NaCl to a final conc. of 0.2 - 0.4 M. The vesicles were then left at 4°C for 2 h prior to centrifugation for 2 h at 230, 000 g. Such treated vesicles were stored at 4°C at a protein conc. of 1mg/ml in the above buffer containing 0.02% sodium azide, 0.2 mM PMSF and 1 mg/ml peptstatin A and used within 4 days.

(vii) Detection of toxin-induced changes in synaptic vesicle proteins.

Toxins were reduced for 30 min at 37°C with 10-20 mM DTT just prior to their incubation with SCVs. At the same time or for a subsequent 60 min period, some toxin samples were incubated with protease inhibitors or a substrate-derived peptide [see (iv) above] before incubation with SCVs for various times and temperatures; 0.2-0.4 mM NaCl was included in this final incubation. Several toxin concentrations were employed and samples were, therefore, adjusted such that they all contained the same amount of DTT or inhibitors. The incubation was terminated by addition of SDS-sample buffer followed by boiling for 2-5 min. The vesicular proteins were then seperated by SDS-PAGE using various % acrylamide gels and detected by staining (silver or Coomassie blue) and by Western blotting (see below). Quantitation of results was achieved by densitometric scanning using a Hirschmann Elscript 400 densitometer linked to a 386 PC; such scans were normalized using densitometric values for vesicular proteins which are unaffected by the toxins (see below). Western blotting was carried out by electrotransfer onto PVDF (Immobilon P) membranes. After blotting, membranes were washed twice in 20 mM Tris-HCl pH 7.5, 150 mM NaCl (TBS) containing 0.2% Tween-20, followed by a 60 min blocking step with TBS containing 3% BSA. Finally, blotted membranes were incubated overnight at room temp. with relevant antibodies (either rabbit polyclonals or mouse mAbs). Several washing steps were carried out at various stages and these used TBS containing 0.2% Tween 20 and 0.2 % BSA. The first antibody was then detected by conventional procedures employing second antibody linked to alkaline phosphatase (detailed in **Cenci di Bello *et al.* 1993**) or by the ECL system of Amersham; ECL films were exposed for several times so that various amounts of antigen could be detected in the linear range of the film and quantitated by densitometric scanning.

(viii) Estimation of synaptosomal transmitter release from LDCVs and SCVs.

NA can be released from both SCVs at the active zone and LDCVs from outside this region. The use of both K^+ stimulation and a Ca^{2+} ionophore induces secretion from both regions (total release). Release from non-active zone areas only is induced by the ionophore in the presence of 20 mM Mg^{2+}. The subtraction of this latter amount from the total release measurement gives an estimate for the active zone exocytosis of NA (see text and figure legends for further details). The exocytosis of endogenous glutamate was monitored continuously by a flurometric assay. This method couples glutamate oxidation by added glutamate dehydrogenase to the conversion of NADP to NADPH. The production of the latter was monitored in a Perkin-Elmer LS-5B luminescence spectrometer. Data points were collected at 2 s intervals and exported to LOTUS 123 where the traces were averaged and corrected for Ca^{2+}-independent release (**McMahon *et al.*, 1992**). For the measurement of met-enkephalin, samples were treated with 2M acetic acid and boiled to eliminate protease action, prior to radioimmunoassay of the peptide concentration (Verhage *et al.*, 1992).

(ix) Preparation of pancreatic acinar cells, their permeabilisation and detection of enzyme release.

Acini were prepared from rat pancreas by collagnease digestion, as described previously (Edwardson *et al.*, 1990); aliquots were suspended in permeabilisation buffer (mM: KCl 140; $MgCl_2$.1; Hepes 20 pH 6.5; EGTA 1) containing BSA (1 mg/ml) and streptolysin-O (1.5 mg/ml); appropriate additions were made ($\pm$ 10 μM Ca^{2+}, $\pm$3 mM ATP, $\pm$ 100 μM GTPγS) to give a final volume of 1 ml. After incubation at 37°C for the appropriate period, samples were cooled on ice and pelleted in a microfuge for 1 min. Supernatants were assayed for α-amylase (Rinderknecht *et al.*, 1967) and lactate

dehydrogenase activities (Bergmeyer *et al.*, 1963). The total cell content of these enzymes was determined after solubilisation of cells with 0.2% Triton X-100, and release was expressed as a % of this total. (Values shown with ± S.E., n=3, error bars are not shown where they lie within the dimensions of the symbol). Ca^{2+} was buffered with 1 mM EGTA; free Ca^{2+} concentrations were determined using an iterative computer program based on that of Fabiato and Fabiato (1979).

RESULTS

(i) Evidence for the *Clostridial* toxins being Zn^{2+}-dependent endoproteases.

Zn^{2+} content analysis of BoNT/A, by flame atomic absorption spectroscopy, revealed the presence of 0.9 metal atoms/toxin molecule; likewise, this metal has been found associated with the LC of BoNT types A, E and F and TeTX in a similar stoichiometry (Schiavo *et al.*, 1992 b, c). To determine whether this Zn^{2+} is required for the neuroparalytic activity of BoNT/A and B, at the mammalian motor nerve endplate, the divalent metal ion chelators PTL, DTPA and TPEN were employed. Incubation of BoNT/A or B with PTL prior to their dialysis into Zn^{2+}-free buffer resulted in an extended paralysis time in comparison with that of the control samples which were incubated with buffer only prior to dialysis (Fig. 11A, C). Notably, the addition of Zn^{2+} acetate (50 μM) to the chelator-treated and dialysed toxins resulted in a complete restoration of the activity of both serotypes tested (Fig. 11B, D), indicating that the perturbation of normal toxin function by this chelator was entirely attributable to Zn^{2+} removal. The inevitable presence of Zn^{2+} in the tissue and the surrounding medium increases the likelihood of a certain degree of re-association of the metal with the Zn^{2+}-depleted toxin. To facilitate a more accurate evaluation of the requirement of these Clostridial neurotoxins' for Zn^{2+}, the tissues were bathed in physiological medium containing two additional Zn^{2+} chelators, DTPA and TPEN, prior and subsequent to the addition of BoNT/A or B pre-exposed to PTL. Notably, the activities of these toxins were dramatically hindered by such treatment (Fig. 11A, B) and the delay in the onset of paralysis was markedly more prolonged than observed with the PTL-treated toxins in the absence of DTPA and TPEN. Interestingly, these latter agents were incapable of altering the BoNT/A- or B-induced blockade of neurotransmission unless the toxins had previously been incubated with PTL (Fig. 11A, B). Therefore, it is likely, as also speculated by Schiavo *et al.* (1992b), that the supplementary chelators when used at these concentrations remove unbound Zn^{2+} from the surrounding buffer but are incapable of removing that Zn^{2+} already associated with the toxic proteins.

Additional evidence for the Zn^{2+}-dependent action of BoNT/A and B was provided upon application of a high concentration of Zn^{2+} acetate (30 mM) to the Tris-HCl buffer following exposure of the toxins to PTL and the subsequent removal of the agent by dialysis. The ensuing inhibition of their paralytic behaviour at the neuromuscular junction was extreme (Fig. 11B, D). Incidences of such a depletion of enzymic activity among Zn^{2+}-dependent proteases have been well documented. Proposed mechanisms by which such inhibition occurs include the association of a second Zn^{2+} with a histidine or glutamate involved in catalysis (Holmquist and Vallee, 1974), the binding of a second Zn^{2+} to an inhibitory metal binding site (Mallya and Van Wart, 1989) or the formation of a Zn^{2+} monohydroxide bridge between the carboxyl group of a catalytic glutamate and the Zn^{2+} (Larsen and Auld, 1988). Although it is unknown which of these apply to the toxins, this valuable data lends additional justification to the supposition that the toxin-associated Zn^{2+} plays a catalytic rather than a structural role.

Although providing two vital pieces of information, the Zn^{2+}-dependence and the known HELIH sequence homology between the BoNT types plus TeTX and characterised metallo-endoproteases, these alone do not allow assumption of the mechanism of action for these toxins; in the characterisation of a protease, its susceptibility to selective inhibitors must also be demonstrated. Phosphoramidon and captopril, first exploited for this purpose by Schiavo *et al* (1992a, b) were employed to confirm their metallo endoprotease catalysis at the neuromuscular junction. When added to the nerve hemi-diaphragm preparation with 50 μM bath-applied phosphoramidon, TeTX (10 nM) which had been pre-incubated with this agent (50 μM), was significantly less effective than the control toxin at blocking neuromuscular transmission (Fig. 12A). A similar antagonism of the toxin's action was observed when the phosphoramidon was applied intra-neuronally via liposomes (~100 μM, final bath conc.; Fig. 12A).

Importantly, the delay in TeTX-induced neuroparalysis by these inhibitor-containing liposomes was shown not to be due to the non-specific interaction between the toxin and vesicle membrane-bilayers as liposomes entrapping only the aforementioned buffer did not delay intoxication by TeTX (Fig. 12A). No increase in the delay of TeTX-paralysis induced by either bath- or liposomally-applied phosphoramidon was detected when both were added together (Fig. 12A). A much higher concentration of phosphoramidon bath- (350 μM) plus liposomally-applied (~350 μM) to the tissue with TeTX did not result in a significantly longer paralysis time than that obtained with 50 μM phosphoramidon (Fig. 12A). The effect of phosphoramidon on the inhibitory activity of BoNT/B in this model was also assessed. When 80 pM BoNT/B pre-incubated with phosphoramidon was applied to the diaphragm together with 50 μM of the bath-applied and 100 μM of the liposomally-applied agent, the interval preceding paralysis was significantly longer compared to that following the addition of the same concentration of untreated BoNT/B (Fig. 12B). As with TeTX, this antagonism was not significantly increased by raising the amount of bath and liposomally-entrapped phosphoramidon to a final concentration of 350 μM each (Fig. 12B).

Unlike TeTX and BoNT/B, when 50 pM BoNT/A, which had been pre-incubated with phosphoramidon (50 μM), was applied to the murine tissue in the presence of 50 μM bath or 100 μM encapsulated phosphoramidon, no decrease in the efficacy of the toxin was detected (Fig. 13A). Indeed, only when both un-entrapped (50 μM) and the liposomally-delivered inhibitor (100 μM) were applied simultaneously with the toxin, was any significant delay in the time to botulinisation observed (Fig. 13A). As with TeTX, the latter antagonistic effect was found not to be due to the non-specific binding of BoNT/A to the liposomal membrane as the presence of vesicles containing only buffer were ineffective (data not shown). Therefore, although phosphoramidon was capable of attenuating the latter toxin's action, when compared with the equivalent experiments executed with TeTX and BoNT/B, the effect of phosphoramidon on BoNT/A action was less dramatic. The effect of the agent on the action of this toxin was also not significantly increased by addition of much higher concentrations of the bath and entrapped inhibitor (350 μM of each; Fig. 13A). However, and most importantly, the antagonism of BoNT/A action by this agent was shown to be attributable to the interference of phosphoramidon with the intracellular action of the LC as liposomes containing the latter were significantly less effective at blocking ACh release if prepared with 200 μM phosphoramidon present (Fig. 13B). This confirms that the protease inhibitor interacts with that toxin domain in which the putative active site is found.

The ability of captopril, another metalloprotease inhibitor reported to decrease the activity of TeTX in *Aplysia* and the proteolytic cleavage of Sbr II in SCVs by TeTX or BoNT/B (Schiavo *et al.*, 1992a), to disrupt the blockade of ACh release by TeTX at the mammalian neuromuscular junction was also investigated. When applied to the mouse diaphragm bathed in KR containing 50 μM captopril or 100 μM liposome-encapsulated inhibitor, the efficacy of captopril-pretreated TeTX was significantly diminished in comparison with that of toxin applied in the absence of this agent (Fig. 12C). Addition of 50 μM bath-applied together with 100 μM of the liposomally-entrapped agent, resulted in a diminution of toxin activity equal to that observed when either bath or encapsulated captopril were present (Fig. 12C). Increasing the amount of captopril to 350 μM bath and 350 μM of the entrapped material did not further delay the paralysis by TeTX (Fig. 12C). The effect of captopril on either BoNT/A or B was not assessed and, therefore, cannot be compared.

Despite the measurable diminution of the toxins' activities by the protease inhibitors in the mammalian model, these results have raised certain important points concerning their efficacies. Firstly, BoNT/A was only significantly antagonised upon the addition of bath *and* liposomal phosphoramidon while BoNT/B and TeTX action was effectively delayed by the application of either. This requirement for both extra- and intracellularly-applied inhibitor suggests that the phosphoramidon-BoNT/A interaction is of a lower affinity than the equivalent association with type B toxin or TeTX. Secondly, as the delay in BoNT/B and TeTX activity by bath *or* vesicle-entrapped phosphoramidon could not be extended by adding both simultaneously or by raising the concentration of inhibitor present, phosphoramidon must bind weakly to these serotypes of BoNT and TeTX (the same situation may apply in the case of captopril inhibition of TeTX). This is corroborated by the fact that the quantity of phosphoramidon and captopril required in these experiments is far in excess of that effective on other enzymes; the former inhibits thermolysin with a K_i calculated as 3.4 nM (Komiyama *et al.*, 1975) while the latter blocks angiotensin-

converting enzyme with an IC_{50} of 20 nM (Cushmann and Ondetti, 1980). Although neither K_i nor IC_{50} values have been evaluated for the inhibition of the neurotoxins by either of these agents, it appears that they are more than four orders of magnitude less effective than observed previously with other metallo-endoproteases. It is possible that these most potent neurotoxins have a unique and selective mechanism of substrate recogrition. This may result from a multi-dentate method of binding to the target and or conformational changes in the toxins which reveal otherwise concealed groups in the active sites. At the present time, there is insufficient data available to explain these complex events but as only an incomplete blockade of BoNT/A, B and TeTX activity was obtained with phosphoramidon and captopril, it would be beneficial to evaluate the effectiveness of other established inhibitors of Zn^{2+} endoproteases such as enalapril or thiorphan with the aim of not only discovering clinically-effective antagonists but, also, providing an indicator of the intricacies of their active sites.

The data reported here provides the first evidence that although BoNT/A may act at a distinct and as yet unidentified locus from BoNT/B and TeTX, it also appears to be a Zn^{2+}-dependent endoprotease. Moreover, the liposomal technique was successfully utilised here to transport the two endoprotease inhibitors into mammalian motor nerve endings; hence, upon the discovery or development of more effective inhibitors of these neurotoxins, the promising results achieved here might point towards the application of such technology in the treatment of botulism and tetanus.

(ii) Specific proteolytic cleavage of Sbr in rat SCVs by TeTX and BoNT/B: a substrate-derived peptide and protease inhibitors unveil differences in their enzymic properties.

Characterisation of anti-Sbr antibodies for use in functional studies - By means of affinity purification on a gel containing the peptide used as antigen, antibodies reactive predominantly with Sbr were obtained (Fig. 14). Larger peptides are currently being used as antigens; these are giving even higher titres, affinities and specificities for Sbr.

Sbr is a target for TeTX and BoNT/B but not A - The SDS-PAGE protein patterns of fractions obtained during the purification of SCVs indicates their purity (Fig. 15); it is clear that the SCV marker synaptophysin was greatly enriched with a concomitant large reduction in the number of proteins present relative to the synaptosomal lysate. Gel filtration on Sephacryl S-1000 (**Matsuoka and Dolly, 1990**) removed some contaminating proteins, as revealed by the gel patterns (Fig. 15 A) and the chromotographic trace (Fig. 15 B). In SDS-PAGE the synaptic vesicle protein, Sbr, can be clearly distinguished as a major protein which corresponds to an apparent Mr of 20-21 kDa. When SCVs were incubated with non-reduced toxins, the pattern of vesicular proteins on silver stained gels remained unaltered. Western blots of an equivalent gel probed with rabbit anti-Sbr antibodies and a mouse anti-synaptophysin mAb also indicated that these proteins were unchanged. However, Sbr was proteolyzed selectively if the toxins were reduced with DTT prior to incubation with SCVs. Both TeTX (Fig. 16) and BoNT/B (Fig. 17) cleaved Sbr, unlike BoNT/A (Fig. 16 A); it is only the LC which is a protease, HC being devoid of any Sbr cleaving activity (Fig. 16 A, B). The proteolytic cleavage of Sbr is very specific (see later) as no other proteins on the stained gels appear to be affected; probing with an anti-synaptophysin antibodies indicated that this major vesicular protein is unaffected by the toxins (Fig. 16 and 17). The toxins proteolysed Sbr in a dose-and time-dependent manner (Fig. 16 and 17), with the cleavage occurring at a relatively slow rate. Careful quantitation (see Methods) indicates (Fig. 17) that under the experimental conditions employed about 60% of the Sbr immuno-reactive band can be removed (the antibodies are unreactive with the degradation products. This result accords with the findings of Schiavo *et al.* (1992a), who identified the cleaved protein as an isoform of Sbr, Sbr II; apparently, Sbr I is not proteolyzed readily by the toxin because it contains a different residue at the cleavage site. Thus, the protein remaining represents Sbr I which under most electrophoretic conditions is not separated well from Sbr II.

Salt washing of SCVs allowed detection of cleavage products and demonstration of a subtle difference between BoNT B and TeTX - Under the conditions employed above, cleavage products (12 and 7 kDa bands on SDS-PAGE gels) of Sbr II produced by TeTX or BoNT B were not detectable. Also, the peptide ASQFETS (spans the cleavage site) when incubated for 60 min at 37°C with reduced toxin or constituent LC proved unable to prevent the consequent proteolysis of Sbr in SCVs (Fig. 16B and 17A).

A similar protocol in which toxin was preincubated with 5 mM captopril also failed to affect the subsequent toxin-induced Sbr cleavage (Fig. 16 B and 17 A). These discrepancies with published findings (Shiavo *et al.*, 1992a, b) may be due to endogeneous proteases contaminating SCVs which might proteolyse the breakdown products and the pepide. Consistently, SCVs from the sucrose gradient showed little, if any, Sbr remaining after incubation alone for 2 h at 37°C (Fig. 18 A), indicating the presence of endogenous proteases capable of cleaving this protein and synaptotagmin. However, when such gradient purified vesicles were salt-washed, much more Sbr and synaptotagmin remained after the standard incubation of the isolated organelles (Fig. 18 A), though some degradation persists (Fig. 19 blots; tracks 1 & 2, SCVs incubated for 2 h at 4°C and 37°C, respectively). Clearly, the endogenous contaminating enzyme is not a very specific protease. Thus, this salt-washed preparation could be conviently used to study the action of the *Clostridial* toxins; indeed, reduced TeTX, its LC and reduced BoNT B did proteolyze Sbr in this sample (Fig. 19). Under such conditions immunoblotting studies indicated that the vesicular proteins SV2, synaptotagmin (Fig. 18B) and synaptophysin were not affected significantly.

Also, the specific toxin-induced cleavage products of Sbr could be detected in SDS-PAGE gels by protein staining (two fastest bands) in these salt-washed vesicles under certain conditions (Fig. 19). The Zn^{2+} chelators, EDTA and PTL were able to prevent the action of these toxins on this substrate; preincubation of the toxins with Zn^{2+} after PTL treatment partly restored their proteolytic activity (Fig. 19; Table 5). The Zn^{2+} protease inhibitor, phosphoramidon, failed to block the toxins' induced cleavage of Sbr from these salt-washed SCVs (Fig. 19; Table 5). However, incubating the toxins with very high initial concentrations of the ASQFETS peptide or captopril during the DTT reduction step showed that the action of TeTX could be antagonised (Fig. 19; Table 5). Intriguingly, these two compounds still failed to interfere with BoNT/B action (Fig. 19; Table 5). This is the first indication that the specificities of these two *Clostridial* neurotoxins towards Sbr are not identical. It is clear that these Zn^{2+} protease inhibitors are not particular good drugs to use in the possible treatment of botulism or tetanus because they are very poor inhibitors and work only at 10,000-fold higher concentrations than required for their normal target proteases. Similarly, it would appear that BoNT/B and TeTX have different requirements for recognising their substrate which may involve different amino acid sequences in the Sbr molecule. Current investigations on the behaviour of different size peptides derived from the Sbr sequence should document these subtle dissimilarities, with the long term aim of designing much more effective toxin inhibitors.

(iii) Dissimilarities in the actions of BoNT A/, E and TeTX revealed with a microtubule-dissociating drug and Ca^{2+} ionophore, though all block transmitter release from SSVs and LDCVs.

Intact microtubules are required for optimal activity of all the toxins except BoNT A in blocking synaptosomal release of several transmitters -. There is a requirement for intact microtubules for BoNT/B, but not /A, to exert its full toxicity at an intracellular site in synaptosomes (**Ashton and Dolly., 1991**). As TeTX and BoNT/E or F appeared to be more like type B than A (**Dolly, 1990**), the effect of a microtubule-disrupting drug on their action was investigated. Colchicine pretreatment did partially interfere with the action of TeTX, BoNT/F and unnicked E (Fig. 20 A, D, E) on synaptosomal NA release; although, there was a difference in efficacy between unnicked and nicked E toxin (Fig.20 E insert), colchicine still only partially antagonised the action of the latter (data not shown). Importantly, the fact that most BoNT types have distinct ecto-acceptors [see section 3(i)] reaffirms that colchicine is not simply acting at the level of toxin binding. Because TeTX and BoNT/B seem to act on the same intracellular target in peripheral cholinergic nerve terminals (Gansel *et al.*, 1987) and on isolated synaptic vesicles (see above), it is not surprising that colchicine antagonises the action of both. Furthermore, as the internalization processes for BoNT/B and TeTX are quite distinct (Black and Dolly, 1986a,b; Parton *et al.*, 1988; Manning *et al.*, 1990; **Poulain *et al.*, 1991, 1992a, b**), they are unlikely to be each affected by a microtubule-dissociating drug; instead, this drug acts inside the nerve terminal. Also, if colchicine blocks these separate processes for each toxin then in co-intoxication experiments one would expect a larger antagonism by this agent compared to that of intoxication by the individual toxins. On the other hand, if each toxin acted at a common locus

which is perturbed by microtubule-dissociating agents then no such additivity would be expected. Although, such double intoxication experiments are complicated due to the pharmacokinetics of the individual toxins, the results obtained to date suggest that there is no additivity of the colchicine antagonism. Accordingly, colchincine should interfere with the action of these toxins on any neurotransmitter if this effect bears any relevance to their molecular mechanism. Colchicine counteracted the action of TeTX upon the release of GABA from synaptosomes.(Fig. 20 B); it also attentuated the effect of BoNT/B upon ACh release from synaptosomes (Fig. 21 C). These results suggest that TeTX and BoNT/B inactivate a target in many different terminal types that is sensitive to disassembly of microtubules. Similar findings were duplicated with the use of other secretagogues; 4-aminopyridine (4-AP) more closely mimics the physiological mechanism of terminal depolarization than does elevated K^+ concentration because it produces spontaneous action potentials, involving the repetitive firing due to the opening of Na^+ channels (Tibbs *et al.*, 1989) leading to an increase in Ca^{2+}-dependent secretion in synaptosomes. However, the antagonism by the microtubule disrupting agent of TeTX (not shown) or BoNT/E intoxication was still apparent when release was stimulated in this way (Fig. 20 F); alone, this concentration of 4-AP, a K^+-channel blocker, did not reduce the efficacy of the toxins.

The observed incomplete antagonism of these toxins' activity by disruption of microtubules may suggests that this cytoskeletal component does not play a direct role in their action. However, the drugs used do not act on all the microtubules inside nerves. Indeed, some microtubules in synaptosomes are resistent to colchicine (Gordon-Weeks *et al.*, 1982); many isotypes of tubulin exist in brain with different affinities for colchicine (Banerjee and Ludvena, 1987). Some subtypes of microtubules may act in vesicle transport whilst others serve a role in the architecture of the nerve (Miller *et al.*, 1987); colchicine-sensitive and -resistant microtubules transport different cellular components (Koike *et al.*, 1989). It can be speculated that these *Clostridial* toxins act on all types of microtubules and so they can still participate in secretion even when the colchicine-labile fraction has been removed. The simplest explanation for the toxins' action would be that they prevent synaptic vesicle disattachment from the microtubules and these organelles are then unable to exocytose and release their neurotransmitter content (**Ashton and Dolly, 1991**). Knowing now that these toxins modify Sbr II, this protein may be important in the attachment-/disattachment of vesicles to this cytoskeleton. Interestingly, the Sbr is homologous to an internal repeat of the collagenase tail region of synapsin I, a region that has been implicated in interactions with synaptic vesicles and the cytoskeleton (Trimble *et al.*, 1988). Also, the high abundance of proline residues in the amino-terminus domains of all Sbr types (Elferink *et al.*, 1989) suggest that an extended rod-like structure may be required in this region for the proteins' appropriate function. Similar proline-rich sequences have been reported for the cytoplasmic domains of synapsin and synaptophysin. The small size of the protein suggests that it is unlikely to have a catalytic function and it may be well suited to function in vesicle transport or membrane fusion. If such a protein played a dual role in attaching vesicle to various components inside the nerve terminal [similar to certain microtubule-associated proteins which link microtubules and organelles to various other cellular components (Severin *et al.*, 1991; Luo *et al.*, 1990; Donato *et al.*, 1989)] including the fusion apparatus (Söllner *et al.*, 1993), then these *Clostridial* toxins could still act at the other loci when microtubules are removed. Studies carried out on the involvement of microtubules in secretion from non-neuronal cells suggest that there is a very highly ordered distribution of various membraneous organelles and vesicle translocation towards the plama membrane (Shimada *et al.*, 1988; Parczyk *et al.*, 1989). Disruption of the microtubular cytoskeleton could alter the directionality of the secretory vesicles (Parczyk *et al.*, 1989) and may even interfere with the final maturation step of these organelles (Herman *et al.*, 1989). Therefore, all *Clostridial* toxins except BoNT/A may act on the synaptic vesicles at an exact site within the nerve terminal which is perturbed by the microtubule disrupting agents; thus, the uniqueness of action of type A deserve further studying.

Elevated intra-synaptosomal Ca^{2+} levels reverse the inhibition exerted by these toxins to varying extents (BoNT/A>>TeTX>>BoNT/E)- In view of the aforementioned dissimilarities between BoNT/A and the other *Clostridial* toxins, it was pertinent to ascertain if they all behaved differently from BoNT/A with respect to reversal by A23187. Having established dose response curves for the individual toxins (Fig. 10), supra-maximal doses of each were used to intoxicate synaptosomes. Fig.21 A indicates that only type A intoxication can be fully reversed with A23187 even though the amount employed (750 nM) was ~4-fold

higher than required to produce maximal inhibition. In contrast, only partial reversal (35-40% of the control) was seen of TeTX-induced blockade of NA release (Fig. 21 A), a level similar to that found with BoNT/B or F. Type E gave the most striking result in that even less recovery could be obtained, reaching a plateau at 20-25% of the control value (Fig. 21 A). Such a result could have arisen because E is by far the most potent inhibitor of NA release (see Fig. 10 A); 8-80 times less BoNT/E (6-60 nM) than used herein produced almost the same blockade of K^+-evoked NA secretion, yet a similar minimal reversal was seen with A23187. Collectively, our results accord with the data for ACh release at the motor endplates; namely, in both cases the effects, of BoNT/B and F or TeTX can only be overcome partially by increasing the intracellular Ca^{2+} concentration, whereas type A blockade is readily reversed (Gansel *et al.*, 1987). Although our data with E toxin suggests that its intoxication is the least reversible and is, therefore, more like BoNT/B, F and TeTX, there remains the possibility that one was observing a very advanced state of intoxication like the late stage of botulinisation with A at the neuromuscular junction (Molgó *et al.*, 1987). Clearly, use of additional criteria will be necessary to determine the complete spectrum of actions of E.

None of the data found with synaptosomes agrees with those from experiments on neurosecretosomes (Halpern *et al.*, 1990) and hormone secretory cells (Knight *et al.*, 1985; Marxen and Bigalke, 1991) where BoNT/A-induced inhibition of secretion could not be counteracted except when nicotine or veratridine were used (Marxen and Bigalke, 1991) (but this is Ca^{2+}-independent). Thus, the differences between these models were examined [see 4(iv) below], particularly because all these endocrinal preparations release hormone from less specialized regions of the cell than the well defined active zone of synapse-forming nerve terminals.

All the toxins inhibit transmitter release from SSVs and LDCVs in synaptosomes at active and non-active zones- Active zones contain a high density of Ca^{2+} channels (Robitaille *et al.*, 1990) and upon stimulation they allow the Ca^{2+} concentration to reach 100 μM in the vicinity of this region (only 100 nm from plasma membrane) (McMahon and Nicholls., 1991). The latter can be blocked by use of high Mg^{2+} concentration whilst the release of LDCVs can still be triggered simultaneously by K^+ depolarisation and, or ionophores that raise the bulk cytosolic Ca^{2+} level; subtraction of non-active zone release from the total (measured under normal K^+ stimulation), gives a measure of active zone secretion. High conc. of BoNT/E, A and TeTX completely blocked the small amount of non-active zone release apparent in the absence of ionophore (Fig. 21B). This is consistent with the abilities of BoNT/A, /B and TeTX to block the release of met-enkephalin from synaptosomes (see below), a peptide that is secreted from LDCVs outside the active zones. These results also correlate with the toxins' inhibition of exocytotic release of vasopressin and oxytocin from neurohypothesis (Halpern *et al.*, 1990) and their action on the non-synaptic secretion from endocrine cells [see section 4(iv)]. Increasing A23187 concentration (and therefore the level of intracellular Ca^{2+}) augments this type of secretion and partially reverses the action of all these toxins. In fact, the ionophore may be inducing some extra non-active zone secretion that is insensitve to the toxins. The lack of Ca^{2+} induced reversibility of chromaffin cell intoxication by these *Clostridial* toxins may be due to the fact that these cells are unable to induce this extra release from non-active zones (see above). Notably the toxins' inhibition of exocytosis from outside the active zones may help explain the puzzling phenomenon that BoNT/A can block spontaneous, non-quantal release of ACh (Vyskocil *et al.*, 1983; Gundersen and Jenden, 1983). The latter can be detected post-synaptically as a slight depolarization (Vyskocil, 1985), and appears to represent neurotransmitter leakage. Careful electrophysiological experimentation has been unable to detect this type of release from the active zones of nerve terminals (Merriney *et al.*, 1989). However, exocytosis of ACh containing SCVs can occur outside the active zone (Ceccarelli *et al.*, 1988; Vizi *et al.*, 1983), releasing transmitter into the intracellular space. Although the ACh would be released as discrete packages, the amount reaching the receptors would vary and, thus, release could appear to be non-quantal in nature. Such an interpretation also leads to the conclusion that BoNT types are specific probes for exocytotic secretion only.

Fig. 21 A, B represent increases in NA release triggered by equivalent rises in intracellular Ca^{2+} concentration; subtraction of these yields values for active zone release (Fig. 21C). Increasing the intracellular Ca^{2+} level with as little as 1 μM A23187 can nearly completely overcome the action of BoNT/A on the active zone release of NA whilst much higher Ca^{2+} concentration only partially reversed the action of TeTX on this type of release (Fig. 21C). BoNT/E blocked active zone NA secretion almost

completely and A23187 could not antagonise this (Fig. 21C). Again these findings on active zone release are comparble to those reported at the neuromuscular junction (see above). Thus, it would appear that the inhibition of active zone release produced by the various toxins differ in the levels of reversiblilty, suggesting that these observations are not due to non-specific effects of the Ca^{2+} ionophore; otherwise, one would expect that the inhibitory actions of all the neurotoxins would display the same characteristics.

Toxin inhibtion of Ba^{2+}-evoked synaptosomal release of fast and slow transmitters- Ba^{2+} can substitute for Ca^{2+} in supporting depolarisation-stimulated release of transmitters from synaptosomes. Ba^{2+} enters the terminals on depolarisation through voltage activated Ca^{2+} channels, but interacts only weakly with calmodulin. These toxins inhibit Ba^{2+}-dependent release of glutamate (Fig. 22) but the extent of inhibition was lower than the corresponding values for Ca^{2+}-dependent release (for TeTX, BoNT/A and B, 60 (±10), 59 (± 13) and 62 (±16), % respectively). Therefore, it appears that their neurotoxicity does not arise solely via a calmodulin mediated mechanism. In contrast to glutamate release, no difference could be detected in the ability of TeTX to inhibit Ca^{2+} or Ba^{2+} evoked release of met-enkephalin; this demonstrates further diversity in the mechanism of this amino acid and neuropeptide release from SCVs and LDCVs, respectively. These results raise an interesting general question about the role of Ca^{2+}-calmodulin mediated interactions in the release process. In addition to triggering exocytosis Ca^{2+} interacts with calmodulin and, hence, activates a range of enzymes including, Ca^{2+}-calmodulin dependent protein kinase II (Ca-CaM kinase II). It is well established that KCl-depolarisation of synaptosomes causes a transient Ca^{2+}-CaM kinase II phosphorylation of the terminal specific protein, synapsin I. This phosphorylation has been implicated in the regulation of vesicle exocytosis. In the presence of $Ca^{2+}$and Ba^{2+}, the kinetics of KCl-evoked glutamate release are identical, even though in the latter condition there is little or no depolarisation-evoked phosphorylation of synapsin I. In light of the discovery that Sbr II is the TeTX/BoNT target, these studies indicate that its function is not directly modulated by a Ca^{2+}-CaM, whilst the reduced efficacy of these toxins on the release of a fast trasmitter in the presence of Ba^{2+} points to a divalent cation-dependent event being involved to some extent in these toxins' inhibitory action.

(iv) Benefits of employing permeabilisation methods for investigating the toxins action in non-neuronal cells.

Exocytosis from streptolysin-O-permeabilised pancreatic acini cells is resistant to BoNT/A - an unique feature of this process in exocrine cells- The time course and Ca^{2+}-dependency of α-amylase release from streptolysin O-permeabilised pancreatic acini, a model system well described elsewhere (Edwardson *et al.*, 1990), is shown in Fig. 23A. Release from permeabilised acini was nearly complete within 15-20 min after Ca^{2+} addition, but when Ca^{2+} was added at intervals after permeabilisation the secretory response diminished i.e. 'run down' (Fig. 23B), with response being almost completely lost after 10 min. This is most likely due to the leakage of cytosolic proteins which are essential components of the secretory machinery, supported by the observation that the amount of released lactate dehydrogenase, a cytosolic marker, increased with time (Edwardson *et al.*, 1990). In our experiments, the enzyme level detected in the supernatant was not affected by the presence of Ca^{2+} or ATP (data not shown). Under optimal conditions established for measuring Ca^{2+}-dependent amylase release (permeabilisation and stimulation for 20 min at 37°C), BoNT/A was found not to significantly affect release (Fig. 23C); up to 100 nM toxin was ineffective (data not shown). No effect on basal release of amylase was observed at any concentration (Fig. 23C). To test the possibility that BoNT/A might be causing a delay in release, obscured by measuring release at only one time point, the time course of release in the presence of 50 nM toxin was examined. The data shown in Fig. 23D shows that the toxin does not cause a significant delay of amylase release at any time. The precise amount of time required for the toxin to diffuse into the permeabilized acini and exert its pharmacological action is unknown. It could be argued that no block in secretion is seen because the lag period is too similar to the time course of exocytosis. However, Bittner *et al.*, (1989) have shown, in digitonin-permeabilized adrenochromaffin cells, that pre-incubation of cells with toxin prior to challenging with Ca^{2+} does not enhance the inhibition of Ca^{2+}-dependent catecholamine release observed. It occurred to us that since BoNT/A exerts its toxic action in cholinergic nerve endings after entering neuronal cells via receptor-mediated endocytosis (Dolly *et al.*, 1984; Black

and Dolly, 1986b and see above), passage through a low pH (endosomal) compartment may be important for the action of BoNT/A. A protocol was employed which exposed toxin pre-bound to the cell surface to low pH (Sandvig and Olsnes, 1981); however, such pretreatment did not significantly enhance the activity of the toxin (data not shown).

This protocol for Ca^{2+}/ATP-dependent secretion may have obscured the effect of the toxin on a particular component of release. The data shown in Fig. 24A reveals, however, that 50 nM BoNT A had no effect on exocytosis evoked by 10 μM Ca^{2+} alone, or on the components of release stimulated by 3 mM ATP or 100 μM GTPγS. Finally, following reports that in chromaffin cells, BoNT LC must be dissociated from the HC for it to exert its intracellular action (Stecher *et al.*, 1989), the effect of LC was tested but again no change was noted (Fig. 24B). This lack of toxin action on secretion from an exocrine cell contrasts with effects of *Clostridial* toxins seen in permeabilized adrenochromaffin (see below) and PC12 cells (**McInnes and Dolly, 1990**), models for regulated secretion from neuroendocrine cells. It is interesting to note that our results are in concurrence with those published recently by Stecher *et al.* (1992). Clearly, there is a marked difference in the action of BoNT on regulated exocytosis in cells of neuronal and non-neuronal origin. While the evoked catecholamine release from chromaffin cells and PC12 cells occurs over a much slower time course than that seen in neuronal cells, in both cell types release is evoked by influx of extracellular Ca^{2+} through membrane channels in response to a hormone or electrical stimulus. Interestingly, release from acini is normally evoked by an extracellular hormonal stimulus which induces an increase in cytosolic Ca^{2+} from intracellular stores; plasma membrane voltage-dependent Ca^{2+} channels are not involved (Petersen, 1992). This may be a crucial difference in the secretory machinery of exocrine cells compared to cells of neuronal origin, especially in view of the apparent association of voltage-dependent Ca^{2+} channels with synaptic vesicle (synaptotagmin) and plasma membrane (syntaxin, neurexins) proteins believed to be involved in exocytotic fusion (Leveque *et al.*, 1992; Bennett *et al.*, 1992). It does, however, remain to be established whether the lack of effect of BoNT/A in the exocrine cells is a consequence of the exocytotic components having diverged during evolution.

BoNT/A and TeTX block ATP-dependent but not ATP-independent exocytosis from digitonin-permeabilised bovine adrenochromaffin cells- Digitonin-permeabilised chromaffin cells provide an excellent model for determining the molecular requirements for exocytosis. The detergent-induced pores allow rapid entry and exit of molecules up to at least 150 kDa in size but the cells retain a good secretory response to a Ca^{2+} stimulus (reviewed in Burgoyne, 1991). This has allowed study of the effects of cytosolic constituents leaving the cell, re-addition of leaked cytosolic proteins in a purified and concentrated form (Morgan and Burgoyne, 1992) and introduction of foreign agents from the permeabilisation medium (Ali *et al.*, 1989; Schweitzer *et al.*, 1989). Such research has been facilitated by inhibition of the secretory process by BoNT or TeTX which have little effect on intact chromaffin cells (over incubation periods of several hours) but enter permeabilised cells and perturb secretion after short-term incubation with relatively low toxin concentrations (Bittner and Holz, 1988, Bittner *et al.*, 1989a). Secretion in the latter was dissected into MgATP-independent and MgATP-requiring stages, with similar findings have been reported for secretion from PC12 cells (Hay and Martin, 1990). In chromaffin cells, the energy-independent component of release was attributed to granules which had already undergone an MgATP-requiring priming step and, thus, only required an elevation in local Ca^{2+} to trigger fusion and subsequent exocytosis. This represents the earliest component of stimulated release detectable from permeabilised cells, observable within 5 s of Ca^{2+} application and completed within 5 min. The later component of release begins after a short lag, is absolutely dependent on the presence of MgATP and proceeds for over 15 min. Herein, the susceptibility of each component to inhibition by BoNT/A and TeTX LC was investigated.

Cells permeabilised with 20 μM digitonin secreted catecholamine when challenged with 10 μM Ca^{2+} in the absence of exogenous MgATP (Fig. 25, open circles); however, the level of release was greatly enhanced by inclusion of the nucleotide complex in both the permeabilisation and the stimulation buffers (Fig. 25, closed circles). The amount of catecholamine released was dependent on the time elapsed between the addition of the permeabilisation buffer and its replacement with the 10 μM Ca^{2+} stimulation medium. The decreasing level of secretion has been termed "run-down" and was attributed to

the leakage of components involved in the exocytotic process. As expected, the inclusion of the nucleotide complex in medium bathing permeabilised cells counteracts the loss of endogenous nucleotide through the membrane pores and allows the priming process to continue after permeabilisation. Exposing cells to BoNT/A or LC of TeTX, at the same time as the permeabilising agent, resulted in a blockade of the MgATP-requiring components whilst being ineffective against release from primed granules (Fig. 25). The results indicate that both toxins act at some site(s) upstream from the Ca^{2+} trigger in the regulated secretory pathway but the incomplete blockade seen with BoNT/A contrasts with the TeTX-induced abolition of ATP-dependent exocytosis; again, this highlights the distinct actions of the two toxins, as revealed by other measures [see 4 (iii)].

CONCLUSIONS

The extensive and multi-disciplinary research in this report has yielded significant amounts of novel results. These have numerous and important implications for both the development of a future clinical treatment of human botulism, and for basic research aimed at deciphering the molecular mechanism of the fundamental process of Ca^{2+} -dependent exocytosis. Throughout the text several of those implications have been outlined in the context of the interpretation of our results. Most importantly, the research programme is on schedule to achieve the major goals of the contract. Already, adequate information has been gained for designing a cholinergic neuronal membrane transporter based on 'inactivated' BoNT/A. Additionally, intracellularly-neutralising mAbs have been obtained; eventually these (in single chain form) could be conjugated to the transporter, followed by evaluation of their targetting ability and neutralising effectiveness. Such achievements in the future would provide 'proof of principle' for the drug targeting hypothesis detailed in our contract. The recent and exciting progress reported on the protease activity of BoNT serotypes has opened up new avenues for the development of additional intracellularly-acting antagonists of these toxins, namely, potent and specific inhibitors of their enzymic activities. Moreover, the importance of their substrate, Sbr II, in exocytosis can now be examined successfully; the resultant information will invariably help us achieve the aforementioned goals of this contract. Thus, in the remaining time, extra effort will be devoted to the latter at the expense of the proposed studies on the molecular definition and functional roles of BoNT ecto-acceptors.

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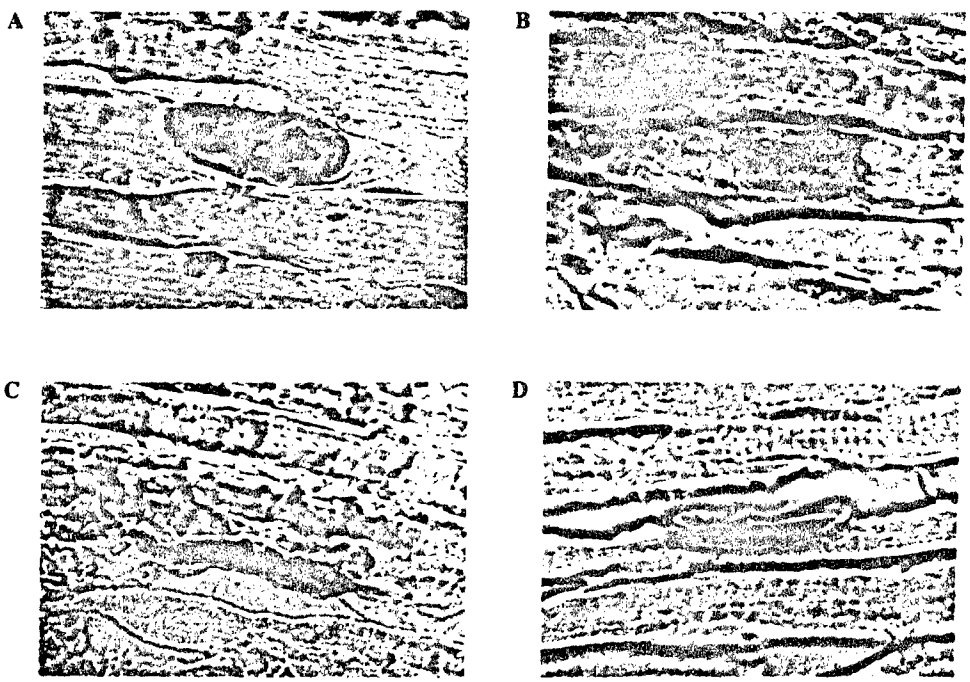


Fig. 1. Saturable binding of BoNT/A colloidal-gold conjugate underlies selective targetting to murine nerve-muscle junctions. Mouse diaphragms were incubated for 15 min at 4°C with toxin-gold conjugate either at 1 nM (A) and with 100 nM unlabelled BoNT/A (B), or at 10 nM alone (C) and with 10 nM unlabelled BoNT/A (D). The diaphragms were then processed for light microscopy; photomicrographs show longitudinal sections. Magnification x 800.

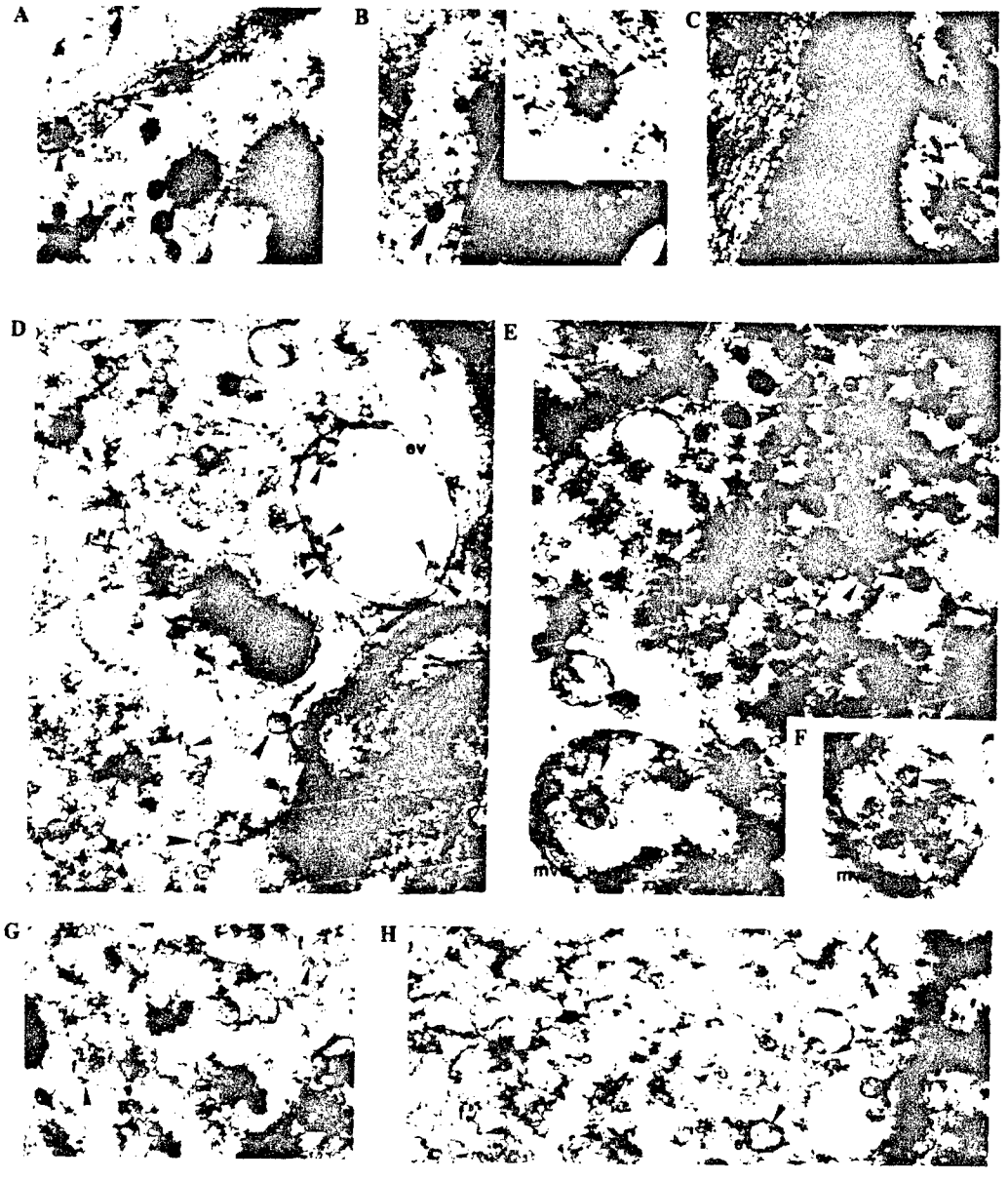


Fig. 2. Electron micrographs of mouse motor nerve endings pre-labelled with BoNT/A gold complex showing particles in endocytotic compartments and cytosolic locations. A mouse diaphragm was incubated with conjugate (10 nM with respect to toxin content) for 30 min at 22°C, washed, fixed, sectioned and processed for EM as described in the Methods [section 2(i)]. In (A) and (B) grains (small arrowheads) are seen at the plasma membrane (pm) and in a coated vesicle (arrow; inset B shows a higher magnification view of coated vesicle and grain). Particles were also seen in large endocytotic vesicles (ev; D, E and H), in multi-vesicular bodies (mvb; E and F) and on filament-like structures (f) in the cytosol (D, G and H) as well as in numerous small smooth vesicles (large arrowheads; D and E). (C) Nerve terminals from a mouse hemi-diaphragm incubated with conjugate (10 nM with respect to toxin content) for 90 min at 22°C in the presence of 100-fold excess of unlabelled BoNT/A. Magnification (A), (B) x 56 000; inset (B) x 150 000; (C)-(H) x 75 000.

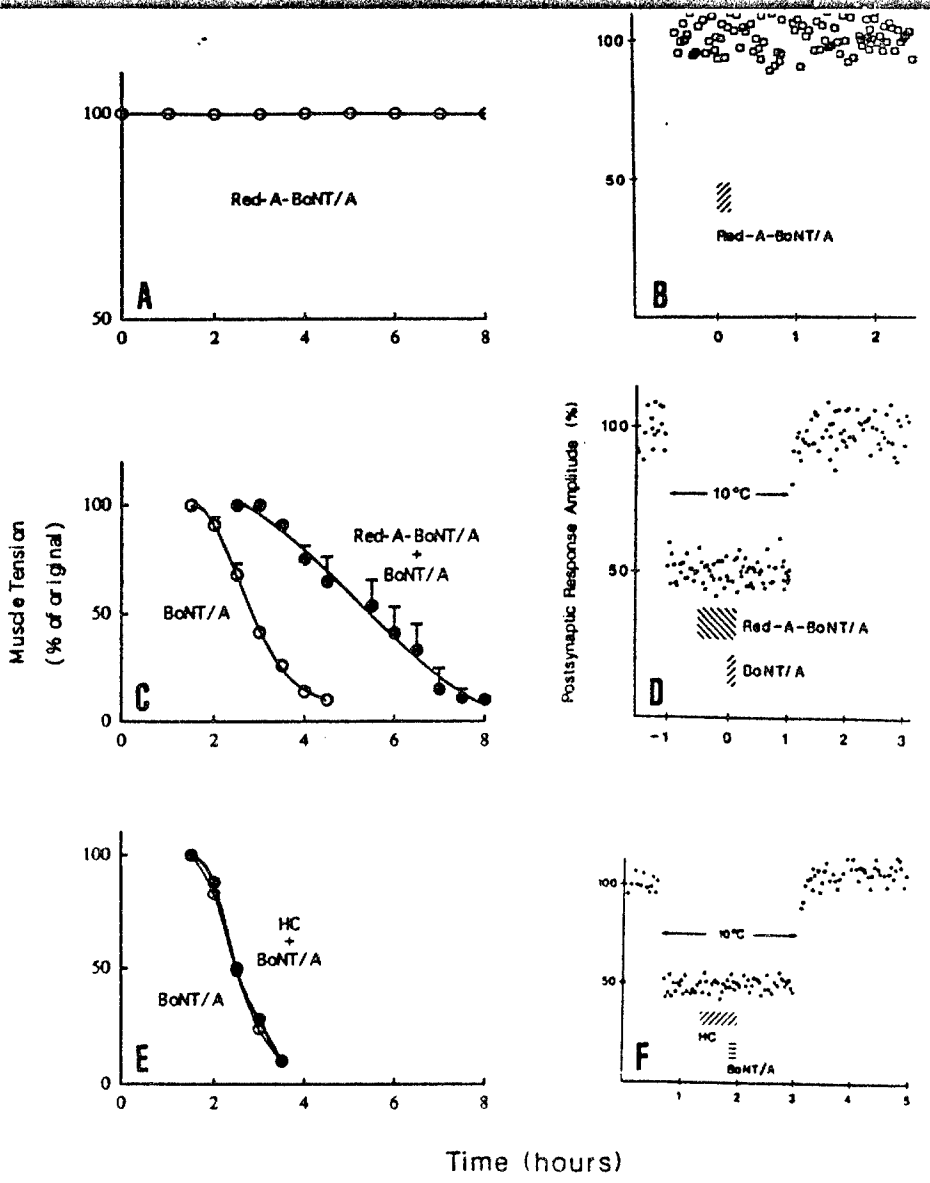


Fig. 3. Albeit inactive Red-A-BoNT/A, unlike HC, antagonises BoNT/A-induced neuroparalysis at murine and Aplysia cholinergic synapses whereas HC is effective only in Aplysia. Bath application of 2.5 nM Red-A-BoNT/A was incapable of altering transmitter release at the mouse phrenic nerve-hemi-diaphragm (A) and *Aplysia* neurons (B). Competition studies undertaken by applying 2.5 nM Red-A-BoNT/A for 45 min to nerve diaphragms under conditions designed to minimise toxin internalisation (see text), followed by a 15 min incubation with 0.2 nM BoNT/A. The toxin and its derivative were removed by thoroughly washing before stimulating the nerve. Under these conditions, Red-A-BoNT/A delayed the onset of the BoNT/A induced paralysis (C); similarly, 100 nM Red-A-BoNT/A applied to *Aplysia* neurons at low temperature for 30 min blocked the action of 10 nM BoNT/A (D). In contrast to the antagonism of BoNT/A action observed with Red-A-BoNT/A at mammalian muscle endplates, the isolated HC of BoNT (30 nM) was incapable of delaying the onset of botulinisation by 0.3 nM BoNT/A (E) under the binding competition conditions described in the text. However, the isolated HC (100 nM) did block the inhibition of ACh release induced by 10 nM BoNT/A in *Aplysia* neurons (F), a discrepancy which, although remaining unexplained, probably results from a less stringent structural requirement for binding at invertebrate toxin acceptors. Adapted from **Maisey** *et al.* (1988); **Poulain** *et al.* (1989); **de Paiva** *et al.* (1993)

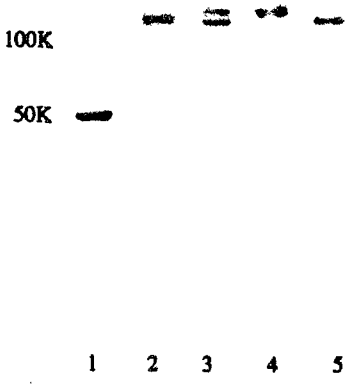


Fig. 4. Different mobilities of Ren-A-HC in SDS-PAGE under reducing and non-reducing conditions reflects the presence of intra-disulphide bond. Electrophoresis was performed on 8% acrylamide gels under non-reducing (*lanes 1-4*) and reducing (*lane 5*) conditions. *Lanes 1*, LC purified from BoNT/A; *2*, HC from BoNT/A in the presence of 10 mM DTT and 2 M urea; *3*, the isolated HC following dialysis into 50 mM Tris-HCl / 150 mM NaCl, pH 8.0 over 24 h and then alkylation with iodoacetamide (150 mM); *4 and 5*, as in *3* but after dialysis into the same buffer for 72 h followed by alkylation. Together with the Ellman's reaction revealing the free thiol content of the Ren-A-HC, prior and subsequent to its further reduction (Table 3), the different mobilities seen in these gels indicate that the intra-chain disulphide was almost completely reformed following renaturation of the HC over 72 h, but as shown in *lane 3*, only partly after 24 h. The protein bands were visualised by staining the gels with Coomassie Blue R-250. Taken from de Paiva *et al.* (1993).

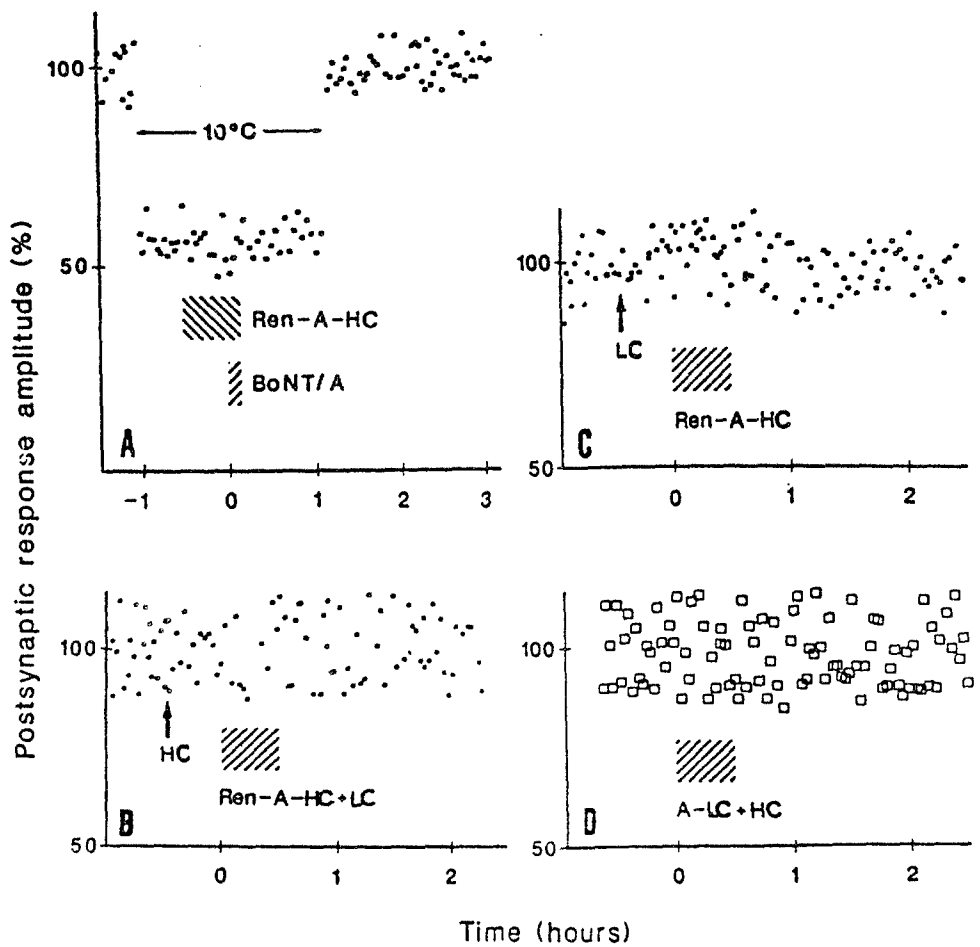


Fig. 5. Alkylated chains of BoNT/A do not undergo internalisation in Aplysia cholinergic neurons. Conditions were as described in Fig. 3. (A) At 10°C, pre-incubation of the ganglion of *Aplysia* with 100 nM Ren-A-HC for 30 min (first hatched area) before the addition of 10 nM BoNT/A (second hatched area) - the internalisation being arrested at this low temperature - prevented inhibition of transmission by the toxin that normally occurs at 22°C. (B) Bath application (hatched area) of Ren-A-H and LC (100 nM each) to a neuron pre-injected with unmodified HC (10 nM, final concentration) did not depress release. (C) When Ren-A-HC (100 nM) was bath applied to a neuron pre-injected with LC release also remained unmodified and (D) the same observation was made when an equimolar mixture of HC plus A-LC (100 nM, each) was applied to the bathing medium. Collectively, these results indicate that the alkylation of either the LC or HC is sufficient to disrupt membrane translocation. Taken from **de Paiva** *et al.* (1993).

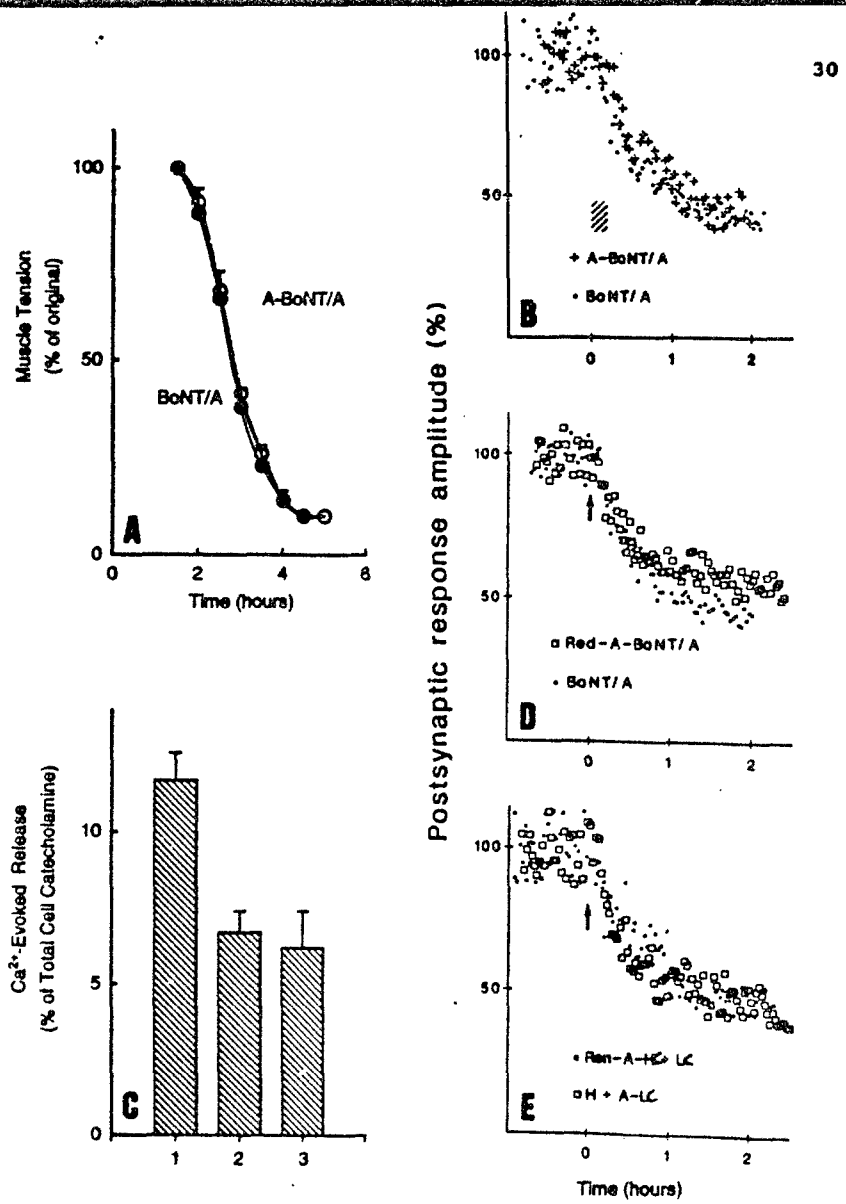


Fig. 6. Free thiols of BoNT are not essential for any phase of its neuroparalytic action in mammals or invertebrates. A-BoNT/A (0.2 nM) was effective as BoNT/A (0.2 nM) in inhibiting ACh release at the neuromuscular junction (A) and 10 nM of this modified BoNT/A (B) was equipotent with the same concentration of native toxin at the buccal ganglion of *Aplysia*. In contrast to its loss of ability to inhibit release when applied externally, addition of 100nM Red-A-BoNT/A to permeabilised bovine adrenal chromaffin cells resulted in a diminution of the normal Ca^{2+}-evoked catecholamine release (1), equipotent (2) to that induced by the same concentration of the native toxin (3) (C) [Note the partial inhibition by BoNT/A, as already reported for this system (Stecher *et al.*, 1989; Bittner *et al.*, 1989)]. Similarly, intra-neuronal injection into the *Aplysia* neuron of 10 nM Red-A-BoNT/A was as effective as 10 nM BoNT/A at inhibiting ACh release (D). Confirmatory evidence that neither the disulphides nor the thiols of BoNT/A are involved in the intracellular activity was provided (E) when injection (arrow) of heterologous mixtures containing one renatured and alkylated chain together with an unmodified chain into a presynaptic neuron (10 nM, final concentration for each chain) induced a depression similar to BoNT/A (see A). Adapted from de Paiva *et al.* (1993).

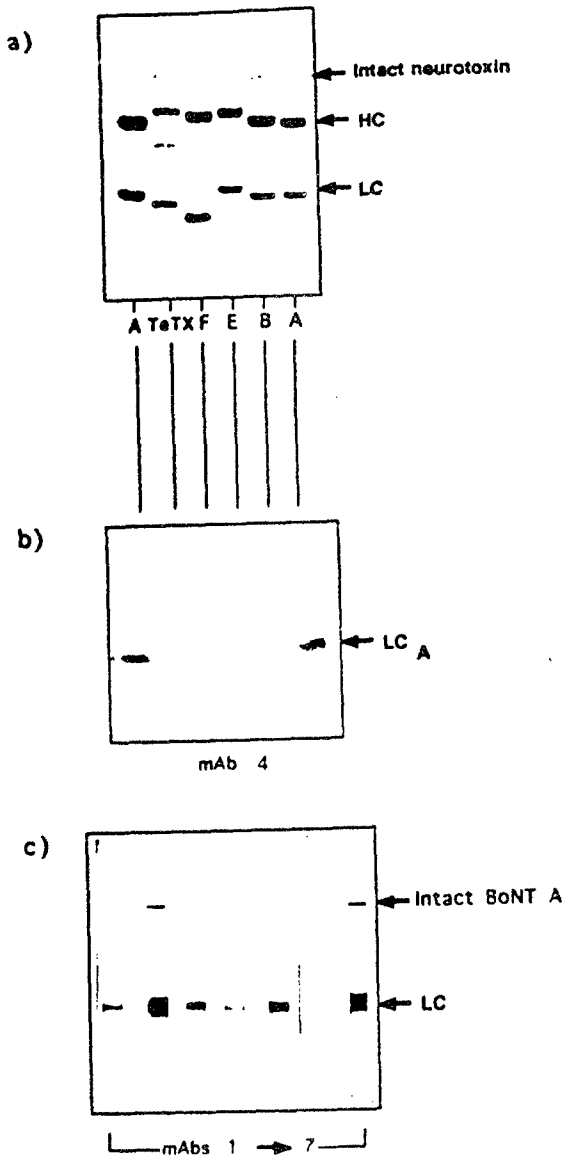


Fig. 7. Exclusive reactivity of anti-LC mAbs for the LC of BoNT/A. Samples of BoNT subtypes A, B, E, F and TeTX in replicate 8% gels were subjected to SDS-PAGE under reducing conditions. Constituents HC and LC of each type of neurotoxin were visualised by silver stain of gel (A) and in the corresponding Western blotted gel, immuno-stained by pre-incubation with each mAb (C). As shown for the representaive mAb 4 (B), reactivities are limited to LC and intact BoNT/A (C). Relative reactivities (dilutions) of the mAbs are indicated in Table 4. Taken from Cesci di Bello *et al.* (1993).

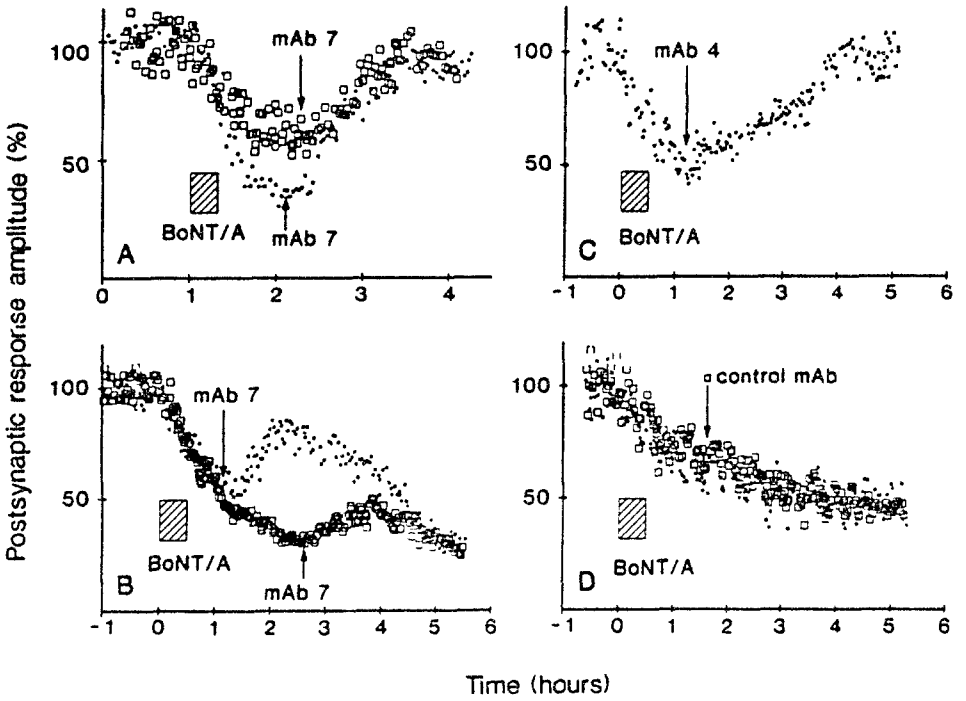


Fig. 8. Blockade of transmitter release induced by BoNT/A is reversed by injection of anti-LC mAbs into Aplysia neurons. (A) After BoNT/A was bath applied [hatched area 10 nM (□) or 30 nM (■)] and 50 % of blockade of neurotransmission ensued, mAb 7 ascitic fluid (■) or purified mAb 7 IgG (□) (final conc. 200 nM) was injected into presynaptic neurons. This was followed by the recovery of ACh release. (B) BoNT/A was bath applied at 25 nM (hatched area), then purified mAb 7 IgG was injected (arrows) into the presynaptic neurons at different times (80 min. or 160 min.) after application of BoNT/A. The intracellular IgG concentration (final) at either time was 170 nM. The extent of recovery of ACh release depended on the time of injection; mAb injected at 80 min. after toxin application produced virtually full recovery but a much lower restoration of activity resulted from injection at the later time of 160 min; recovery was not sustained and transiency was noticeable in experiments performed for longer than 4h. (C, D) After BoNT/A (10 nM) induced a significant blockade of ACh release, mAb 4 ascitic fluid (C) or control ascites (□) (D) or a mAb raised to an unrelated protein (■) were injected into presynaptic neurons; recovery of neurotransmission is seen only in the neuron injected with mAb 4. None of the antibodies exerted any effect on neurotransmission in non-toxin treated neurons. Taken from **Cenci di Bello *et al.* (1993).**

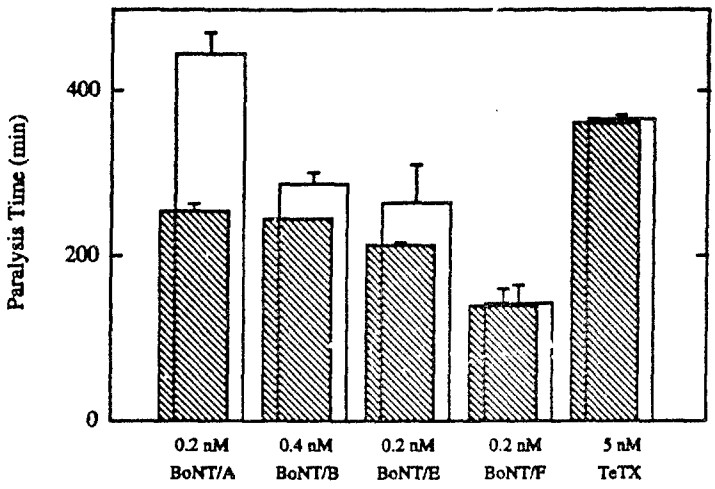


Fig. 9. Red-A-BoNT/A reveals that types B, E, F and TeTX do not share ecto-acceptors with BoNT/A at motor nerve terminals to any significant extent. Binding competition studies were performed as described before. BoNT/A, E, F (0.2 nM), BoNT/B (0.4 nM) and TeTX (5.0 nM) were bath applied for 15 min to the mouse phrenic nerve-hemidiaphragm bathed in MKR at 4°C (hatched bars). Additionally, separate tissues were pre-treated with Red-A-BoNT/A (2.5 nM) for 45 min before the addition of the native BoNTs or TeTX, under these conditions designed to minimise toxin internalisation (empty bars). After thorough washing, the diaphragms were bathed in KR and the temperature raised to 24°C, or 37°C for the TeTX experiment, before stimulation of the nerve. The paralysis time was taken when the evoked muscle tension had decreased to 10 % of the original. All values are the means of at least 3 individual experiments ± S.D. Adapted from Ashton *et al.* (1993).

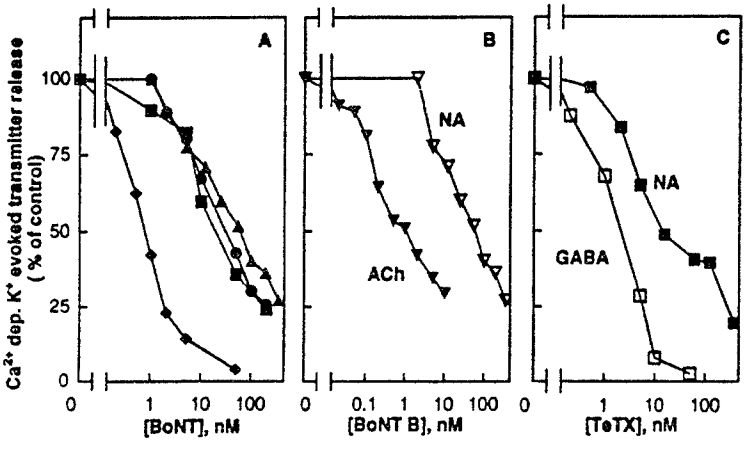


Fig.10 Inhibitory potency of BoNT and TeTX on the synaptosmal release of various transmitters. Synaptosomes were incubated with the different transmitters and the various toxins for 2h at 37°C. The toxin and excess radiolabelled transmitter were removed by washing in Ca^{2+}-free buffer. Evoked release was measured over a 5 min period in buffer containing 1.2 mM Ca^{2+} and 25 mM K^{+}. An equivalent sample was stimulated in the absence of Ca^{2+} and this amount of release subtracted from that in Ca^{2+}-containing medium to yield the Ca^{2+}-dependent release plotted; values are expressed as % of that obtained in the absence of toxin. (A) Block of NA release by BoNT/A (■), B (▲), E (◆) and F (●). (B) BoNT/B inhibition of ACh (▼) and NA (▽) release. (C) The potency of TeTX in inhibiting release of NA (■)and GABA (□). Results for all panels represent the mean of many experiments; error was less than 15 % of the mean for all points. Adapted from **Ashton *et al.*** (1993).

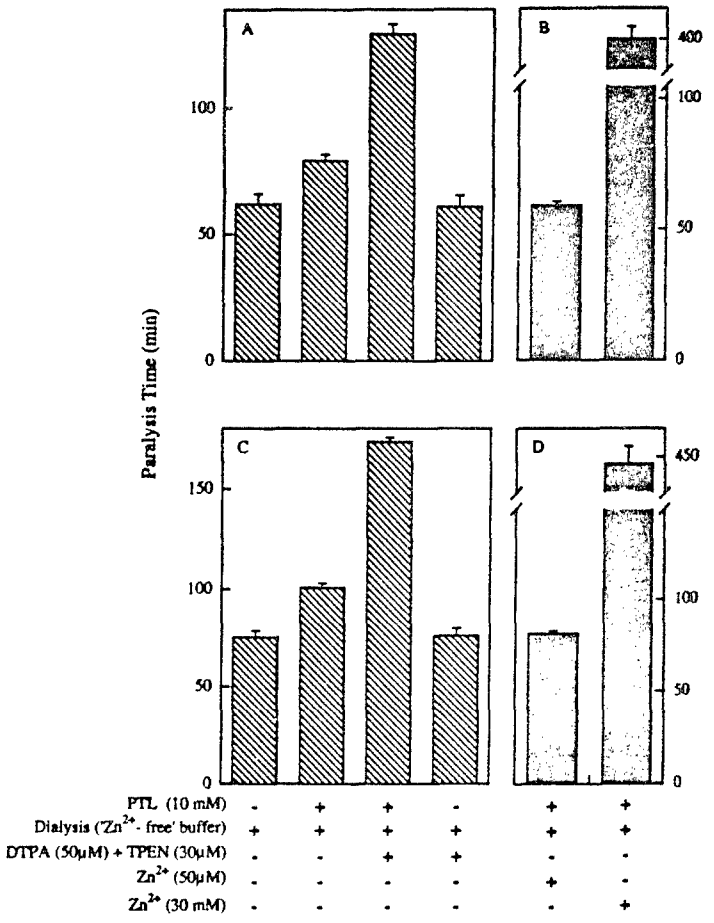


Fig. 11. A low Zn^{2+} concentration is essential for BoNT/A- and /B- induced neuromuscular paralysis but elevated levels are antagonistic in a manner typical of neutral metalloproteases . The times to paralysis ensuing the addition to mouse hemi-diaphragm of 50 pM BoNT/A (A and B) or 80 pM BoNT/B (C and D) pre-incubated for 2 h at 37°C with buffer only (see text for details) prior to its dialysis into 'Zn^{2+}-free' buffer, are plotted in column 1. A measurable delay in time to botulinisation was observed upon addition of toxin pre-incubated with PTL before its dialysis into the 'Zn^{2+}-free' buffer (2). The depletion of BoNT/A or B activity by this chelator was significantly extended upon exposure of the excised nerve-muscle preparation to 50 μM DTPA and 30 μM TPEN for 2 h prior, and subsequent to the addition of the toxin (3); this amount of DTPA and TPEN alone were intrinsically unable to affect the toxin's neuroparalytic activity (4). (B) The effect of PTL on the action of BoNT/A or B was reversed upon the addition of 50 μM Zn^{2+} acetate to the toxin sample following its exposure to PTL and its subsequent dialysis into 'Zn^{2+}-free' buffer (1). In contrast the presence of a high concentration (30 mM) of Zn^{2+} acetate under these conditions resulted in a major loss of the toxin's activity. The paralysis times were measured as the period following the toxin's addition to a 90% depletion of the original nerve-evoked muscle tension and represent the average of three experiments ± S.D. Experimental conditions are summarised in the figure. Taken from **de Paiva and Dolly (1993)**.

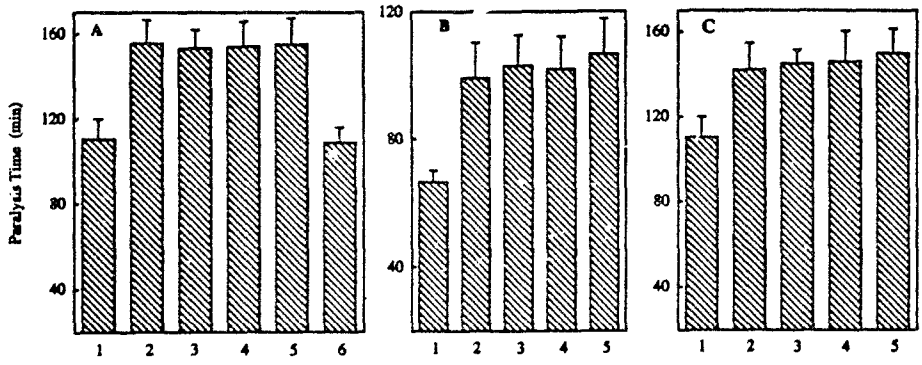


Fig. 12. Diminution by metalloproteases inhibitors of the neuromuscular paralysis activity of TeTX and BoNT/B. (A) When compared to 10 nM control untreated TeTX (1), the neuroparalytic activity of the same concentration of toxin was significantly diminished when pre-treated with 50 μM phosphoramidon and applied to the mouse phrenic nerve-hemidiaphragm together with 50 μM bath-(2), or 100 μM liposomally-delivered phosphoramidon (3), or both simultaneously (4). Raising the concentration of the bath (to 350 μM) and liposomally-applied inhibitor (to ~350 μM) did not further delay the onset of paralysis (5). The possibility of a non-specific interaction between the liposomes and the toxin was eliminated by the observation that the vesicles containing only buffer (see text for details) had no effect on the time to paralysis ensuing the addition of the untreated toxin (6). (B) As observed with TeTX, 80 pM BoNT/B pre-treated with 50 μM phosphoramidon and applied to the murine nerve-muscle preparation with either 50 μM bath-(2), or 100 μM liposomally-administered phosphoramidon (3), or both together (4), was less effective at blocking neuromuscular transmission than the control toxin (1); an increased presence of the inhibitor (350 μM in the bath plus 350 μM entrapped in liposomes) did not further extend this antagonism. (C) The neuroparalytic activity of 10 nM captopril pre-treated TeTX applied to the hemidiaphragm, bathed in medium containing 50 μM captopril (2) or 100 μM liposomally-entrapped inhibitor (3), was markedly diminished in comparison to the same concentration of control toxin added in the absence of inhibitor (1). A similar effect was observed upon the application of both 50 μM bath- and 100 μM liposomally-applied captopril (4) and this was not significantly extended upon raising the amount of inhibitor to 350 μM bath and ~350 μM entrapped captopril in the bath (5). The columns represent the average of at least two experiments ± S.D. Taken from **de Paiva and Dolly (1993)**.

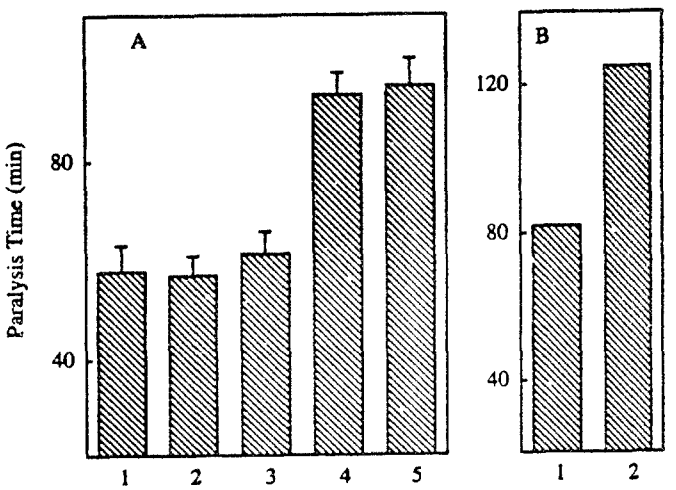
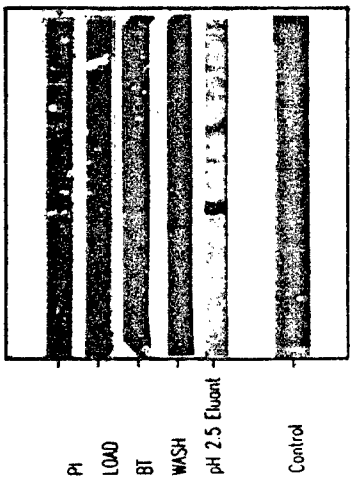


Fig. 13. Inhibition of ACh release by BoNT/A or LC is only attenuated by phosphoramidon when bath-applied and liposomally-delivered into motor nerve endings (A) BoNT/A (50 pM) pre-incubated with phosphoramidon and applied to excised murine phrenic nerve-hemi-diaphragms in the presence of 50 μM bath- (2) or 100 μM liposomal-phosphoramidon (3) was as efficacious as the control untreated toxin applied in the absence of the inhibitor (1). Upon the addition of both 50 μM non-encapsulated and 100 μM liposomal phosphoramidon together with the pre-treated BoNT/A (4), a substantial delay in the time to paralysis was measured. This effect was not significantly extended when the pre-treated toxin was applied with 350 μM bath plus 350 μM vesicle-entrapped inhibitor (5) (B). Liposomes containing the LC of BoNT/A prepared in the presence of 200 μM phosphoramidon (2) were substantially less effective than liposomes containing the same amount of LC but prepared without the inhibitor (1). For panel A, the columns represent the average of at least 3 experiments ± S.D. while the paralysis times shown in panel B are representative of 3 separate liposomal preparations. Taken from **de Paiva and Dolly (1993).**

Visualization by Western Blotting

Bovine SSV



PI = Preimmune serum
LOAD = immune serum
BT= Column Breakthrough
pH 2.5 Eluant = SBR Abs
Control = Abs to Purified SBR protein

Position of peptide r55-70 on Synaptobrevin

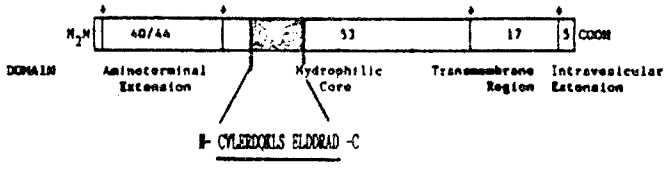


Fig. 14. Production and purification of rabbit antibodies against a Sbr peptide To isolate immunoglobulins (IgGs) specific for Sbr, immune serum was applied to the immobilised peptide resin, followed by salt washing and finally elution of bound IgG using 0.1 M glycine pH 2.5. The different stages in the isolation of the Sbr reactive IgG were monitored by Western blotting using bovine SCVs;. IgG reactive with Sbr can be seen in the pH 2.5 eluant and this has been used to study toxin proteolysis of Sbr.

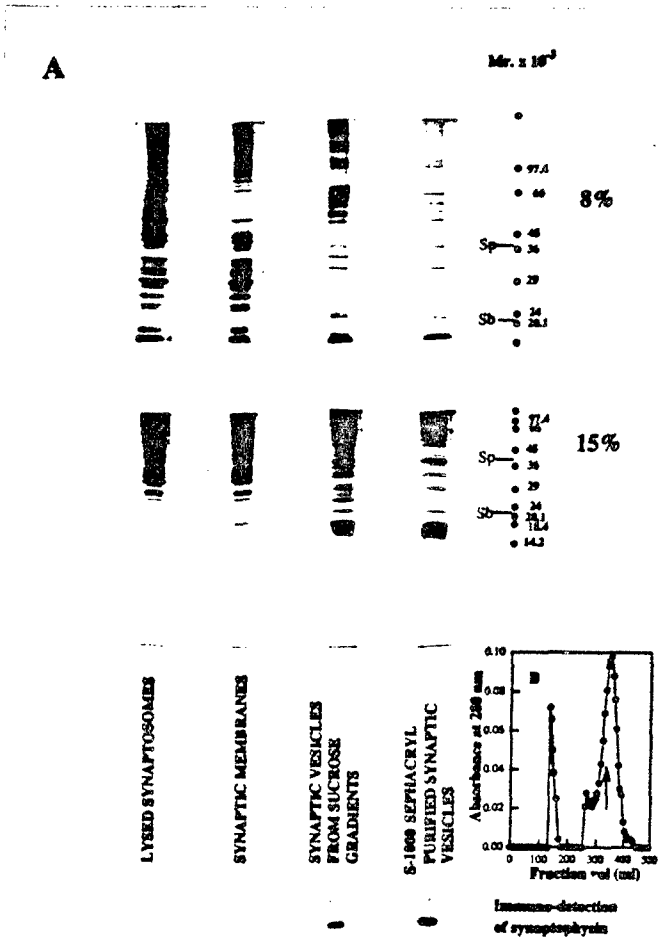


Fig. 15. Enrichment of SCVs from rat cerebrocortical synaptosomes by sucrose gradient centrifugation and gel filtration on Sephacryl. (A) SDS-PAGE silver stained patterns (2 different % acrylamide gels) of proteins present in the particulate material at various stages in the purification of SCVs , and an equivalent immunoblot for a synaptic vesicle marker-synaptophysin- detected with a mouse mAb. Various standards were used [Mr. (kDa): phosphorylase b, 97.4; BSA, 66; ovalbumin, 45; glyceraldehyde-3-phosphate dehydrogenase, 36; carbonic anhydrase, 29; trypsinogen, 24; soybean trypsin inhibitor, 20.1; b-lactoglobulin, 18.4; a-lactalbumin, 14.2] and their positions and those for synaptophysin and Sbr are indicated. The inset (B) shows a typical seperation of SCVs (arrow) from a protein peak in the void volume by chromotography on a Sephacryl S-1000 column.

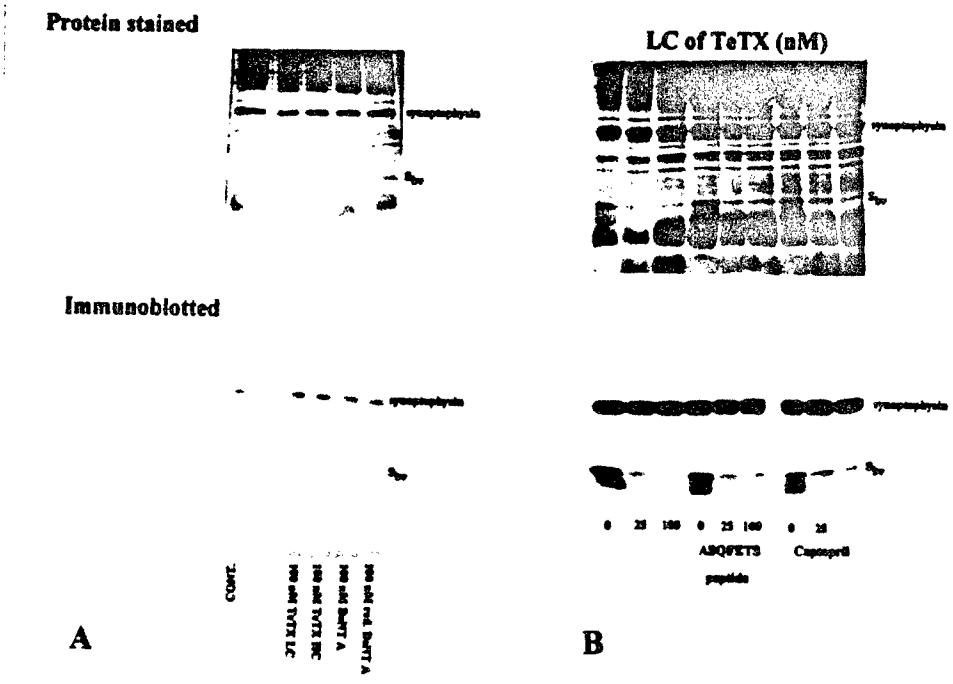


Fig. 16. Sbr in SCVs purified on a Sephacryl column is proteolyzed by LC of TeTX (but not by BoNT A) though this was poorly antagonised by a substrate derived peptide or a Zn^{2+}-protease inhibitor. Purified SCVs were incubated with toxins, or their constituent chains, for 2h at 37°C ; SDS-PAGE (13 % gels) of the samples and corresponiding immunoblots were probed with a mouse anti-synaptophysin antibody (used as an internal control) plus a rabbit anti-Sbr polyclonal antibody and detected using the ECL system. Protein markers for silver stained gels were as described in Fig.15 whilst blots employed biotinylated standards [Bio-rad;Mr.(kDa): myosin, 200; β-galactosidase, 116; phosphorylase *b*, 97.4; BSA, 66; ovalbumin, 45; carbonic anhydrase, 31; soybean trypsin inhibitor, 21.5; lysozyme, 14.4; aprotinin, 6.5]. (A) LC (but not the HC) of TeTX exhibits the proteolytic enzymic acitivity towards Sbr whilst neither intact nor reduced BoNT A cleaved any vesicular protein; 100 nM of each was employed. (B) TeTX LC cleaved Sbr in a dose dependent manner whilst 100 μM ASQFETS (incubated toxin with 360 μM peptide) or 1.4 mM captopril (5 mM initial) had negligible affects on the toxin-induced proteolysis, as also determined by densitometric scanning (see Fig. 17 for further details).

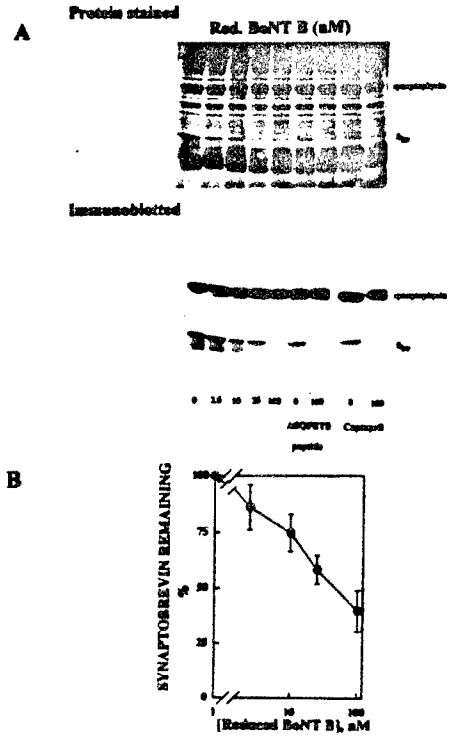


Fig. 17. Dose response curve of BoNT/B-induced proteolysis of Sbr in Sephacryl purified SCVs and minimal inhibition by a Sbr peptide or captopril. BoNT B was reduced with DTT, incubated for 1 h at 37°C with either buffer, 360 μM ASQFETS or 5 mM captopril, prior to additon of SCVs and incubation for a further 2h at 37°C. The amount of inhibitors were adjusted so that there were equal concentrations (100 μM peptide and 1.4 mM captopril) in all relevant samples. (A) SDS-PAGE silver stained gel (13 %) and an equivalent immunoblot (details as in Fig.16). Various amounts of BoNT B were tested, and the effect of the peptide or the protease inhibitor was negligible, as assessed by densitometric scanning of gels and blots. (B) Dose response curve for BoNT B-induced Sbr cleavage . Values were quantitated by densitometric scanning of immunoblots and silver stained gels, followed by normalization (using synaptophysin as an unaffected internal control) and averaging.

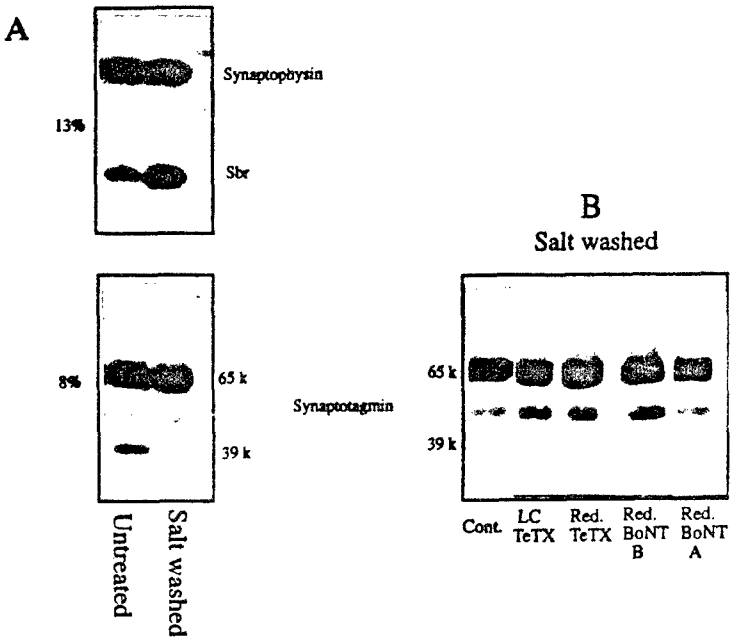


Fig. 18. Salt washing of SCVs from the sucrose gradient prevents breakdown of Sbr and synaptotagmin by endogenous proteases: inability of Clostridial toxins to proteolyze synaptotagmin. SCVs from the sucrose gradient step were diluted in 0.3 M glycine 5 mM Hepes pH 7.4 and 0.2-0.4 M NaCl was added to some aliquots; following a 2h incubation at 4°C, SCVs were pelleted, supernatant removed and vesicles resuspended in buffer. (A) To assess autolysis of vesicle proteins, untreated and salt-washed SCVs were incubated for 2h at 37°C , prior to the seperation of vesicular proteins by SDS-PAGE on 8 or 13 % gels followed by Western blotting. A rabbit anti-synaptotagmin polyclonal antibody was employed whilst other details are as in Fig.16. It is apparent that in non-salt treated vesicles most of the Sbr was proteolyzed by endogenous enzymes (in the presence of the protease inhibitors employed throughout), and that some of the synaptotagmin was cleaved at a reported cleavage site to yield a 39 kDa breakdown product. (B) Salt-washed SCVs were incubated with high doses of BoNT /A/ B and TeTX (or its LC) for 2h at 37°C and SDS-PAGE performed. Synaptotagmin was unaffected by any of these toxins (as shown); similar negative results were also found when another synaptic vesicle protein, SV2, was investigated by immunoblotting (not shown).

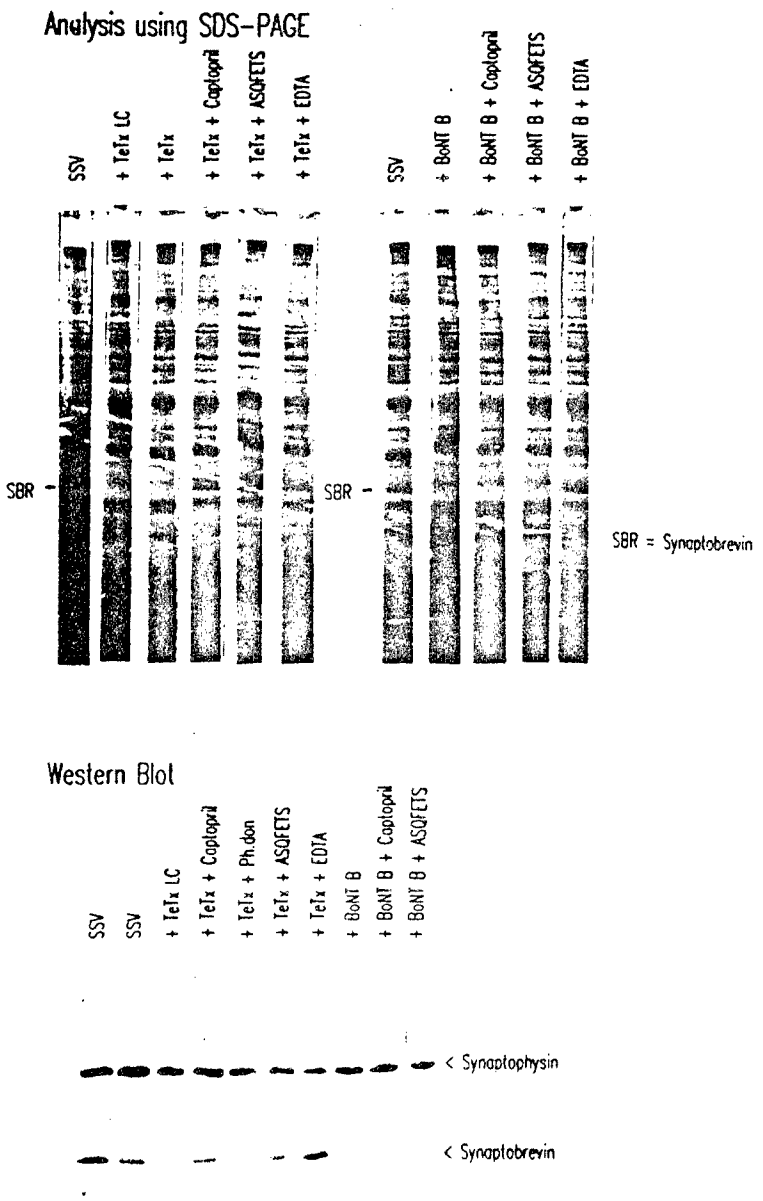


Fig. 19. Selectve cleavage of Sbr in salt-washed SCVs by TeTX or LC and BoNT/B: inhibition of the action of the former but not the latter by captopril and ASQFETS peptide. BoNT/B or TeTX were pre-incubated for 30 min at 37°C with 20 mM DTT in the absence and presence of inhibitors together with captopril (final 1.6 mM), 10 mM ASQFETS (final 1 mM) or 2.5 mM phosphoramidon (final 0.5 mM) before reaction with SCVs for 2 h at 37°C prior to analysis by SDS-PAGE. For samples analysed by SDS-PAGE and Western blotting, 20 and 100 nM toxins were used with 1 and 0.25 ng/ml SCVs, espectively.

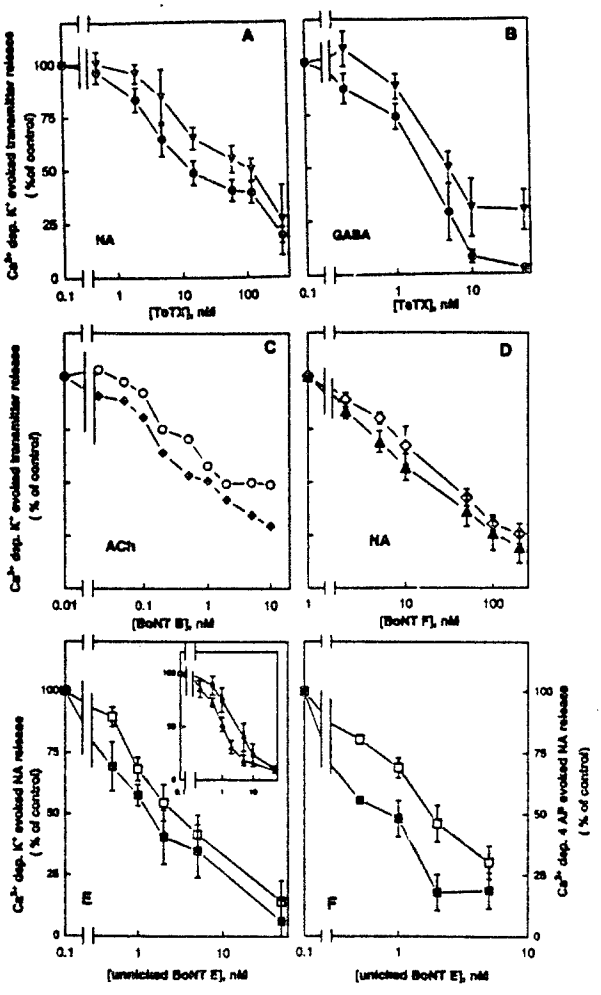


Fig. 20. Antagonism by a microtubule-dissociating agent of the inhibition by BoNT/ B/ E/ F and TeTX of the evoked release from synaptosomes of different transmitters. Synaptosomes were incubated with [^{3}H]NA and treated for 40 min at 37°C with (▼,○,◇,□) or without (●,◆,▲,■,△) 1 mM colchicine prior to an additional 2h incubation in the presence of various toxins. The toxin and excess radiolabelled transmitter were removed by washing in Ca^{2+} free buffers. Evoked release was measured over a 5 min period in buffer containing 1.2mM Ca^{2+} and 25mM K^+. An equivalent sample was stimulated in the absence of Ca^{2+} and this amount of release subtracted from that in Ca^{2+}- containing medium to yield a measure of Ca^{2+} dependent release. Values for secretion are expressed as a % of that obtained in the absence of toxin. All figures indicate the partial antagonism by colchicine of the toxins' action on transmitter secretion. (A)(B) The action of TeTX (▼,●,) on the release of two different neurotransmitters ; results are the mean (+ S.D) of 5 independent experiments. (C) BoNT B inhibition of ACh release (○,◆); values are the mean of 3 experiments. (D) The effect of BoNT F (◇,◆) on NA release (n= 5 expts, + S.D.). Unnicked BoNT E block of 25 mM K^+ stimulated (E) and 0.6 mM 4-AP (F) evoked-NA release, respectively (□,■). Insert in (E) illustrates the action of unnicked (■) or nicked (▲) BoNT E on NA secretion. Type E was fully nicked by incubation with trypsin (final conc. of 10 μg/ml) for 30 min at 37°C before inactivation by the addition of 5-fold excess of soybean trypsin inhibitor. All values were for 3 independent experiments (+ S.D.). Adapted from Ashton *et al.* (1993).

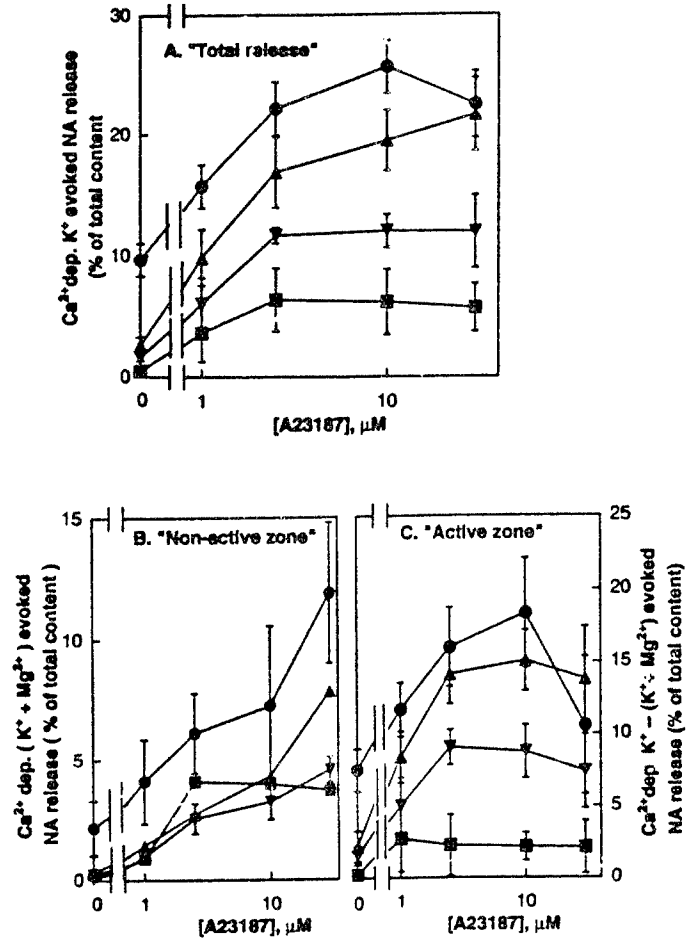


Fig 21. A Ca^{2+} ionopore reveals dissimilarities in the inhibition by Clostridial toxins of Ca^{2+}-dependent synaptosomal NA release from active and non-active zones. Conditions were as outlined in Fig 20 except that no microtubule drugs were employed and the evoked release buffer contained the appropriate conc. of ionophore. Results are means (+ SD) for 3 independent experiments. (▲) 750 nM BoNT/A, (■) 400 nM BoNT/E, (▼) 360 nM TeTX, (●) control. (A) Total release. (B) Non-active zone release determined in the presence of 20 mM Mg^{2+} which prevents Ca^{2+} entering through the Ca^{2+} channels at active zones. The actual amount of ionophore employed was 1.4 -fold higher than plotted so that the exact intracellular Ca^{2+} conc. induced by the ionophore would be equivalent in the presence or absence of 20 mM Mg^{2+} which reduces the ionophore efficacy by this factor (Verhage *et al* , 1991). (C) Values for active zone release were calculated by subtracting the values of Ca^{2+} dep.K^+ + high Mg^{2+} evoked release ("non-active zone' release, B) from the values of Ca^{2+} dep. K^+ evoked release (total release, see A). Compiled from Ashton and Dolly (1991); Ashton *et al.* (1993).

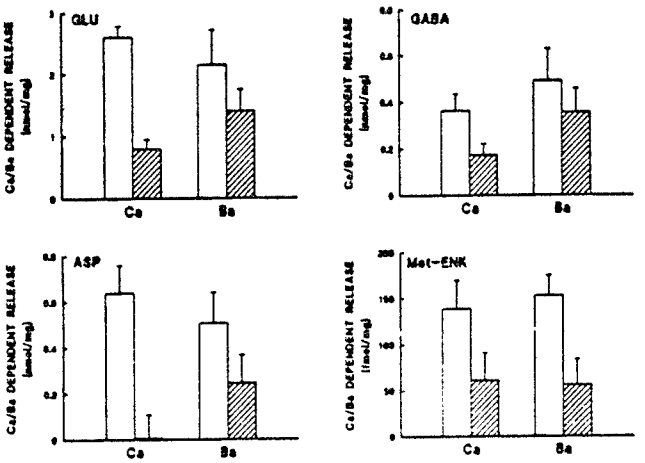


Fig. 22. TeTX inhibition of the Ca^{2+}- and Ba^{2+}-dependent release from synaptosomes of glutamate, GABA, aspartate and met-enkephalin evoked by K^{+}- depolarisation. Guinea-pig cerebrocortical synaptosomes (1 mg of protein/ml) were incubated in the presence or absence of 10 nM TeTX for 45 min (conditions yielding sub-maximal inhibition). Release was initiated by 30 mM KCl in the presence of 1.3 mM Ca^{2+} or Ba^{2+}. The Ca^{2+}/ Ba^{2+} -dependent glutamate (Glu), GABA and aspartate (Asp) release 1 min after KCl depolarisation is shown whereas met-enkephalin release was for 3 min. Amino acids were monitored by derivatisation with o-phthaldialdehyde followed by reverse-phase chromatography and met-enkephalin release was measured by radio-immunoassay. Results are the means of three experiments □, control; ■, 10 nM TeTX. Taken from McMahon *et al.* (1992).

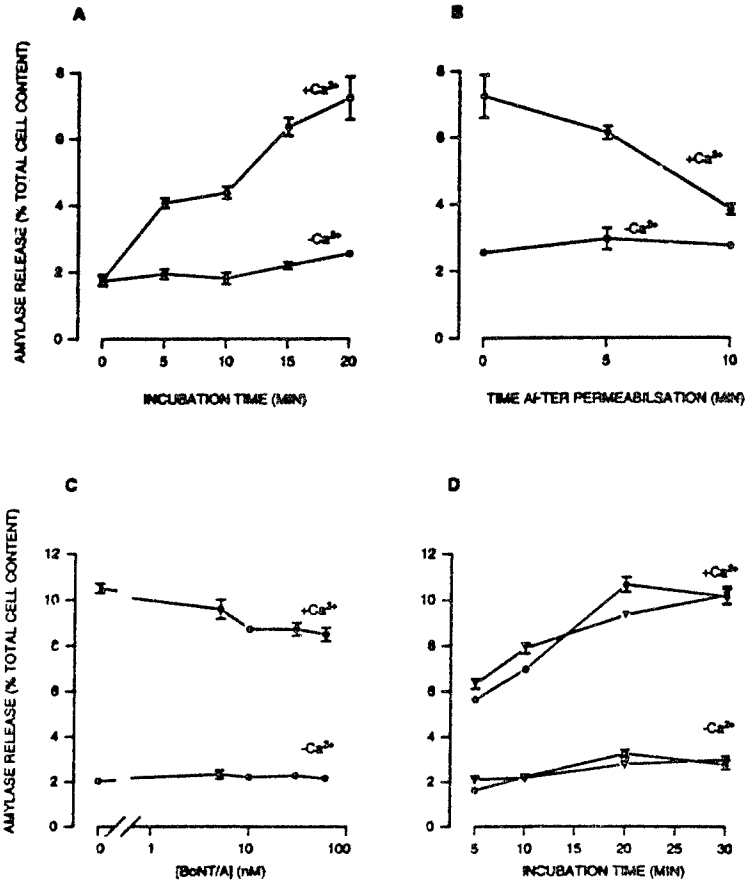


Fig. 23. Exocytosis of α-amylase from pancreatic acinar cells permeabilised with streptolysin-O could not be blocked by BoNT/A. (A) Time course of amylase release from streptolysin O-permeabilised acini incubated at 37°C in the presence of 3 mM ATP with (●) or without (○) 10 μM Ca^{2+}. (B) Run down of amylase release; either 10 μM Ca^{2+} (●) or Ca^{2+} -free buffer (○) was added to pancreatic acini at various times after permeabilisation, followed by incubation for a further 20 min at 37°C. (C) Acini were incubated in the presence of 3 mM ATP with (●) or without (○) 10 μM Ca^{2+} and various concentrations of BoNT/A for 20 min at 37°C. (D) Cells were incubated in the presence of 3 mM ATP with (▼,●) or without (▽,○) 10 μM Ca^{2+}, and with (▼,▽) or without (●,○) 50 nM BoNT/A for various times at 37°C. Values are means ± S.E.

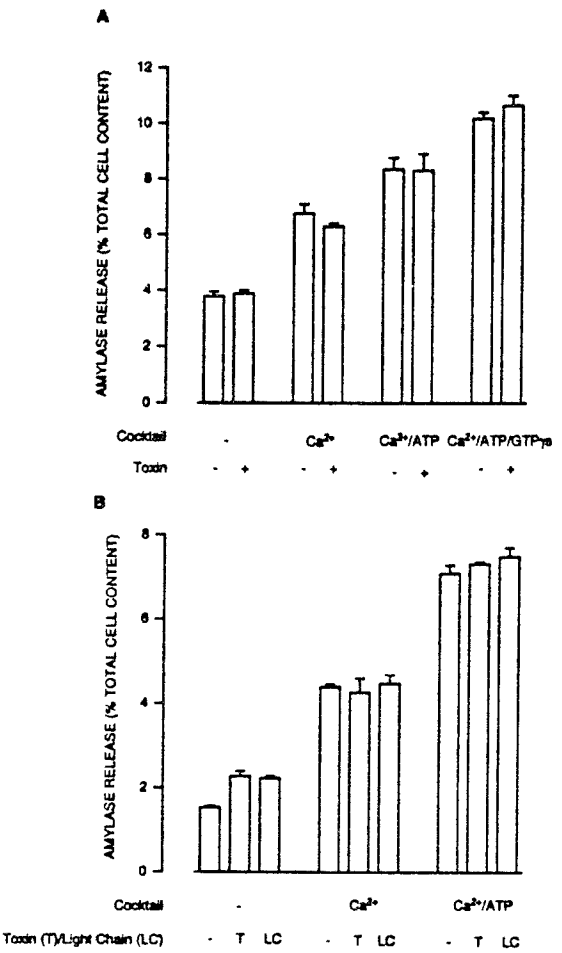


Fig. 24. Lack of effect of BoNT/A and its LC on ATP and GTPγs enhancements of Ca^{2+}-dependent release of α-amylase from permeabilised acini. (A) The streptolysin O-treaed cells were incubated for 20 min at 37°C with 50 nM BoNT/A in the presence or absence of 10 μM Ca^{2+}, with or without 3 mM ATP and 100 μM GTPγs. (B) Acini were incubated for 20 min at 37°C with either 50 nM BoNT/A or 50 nM LC in the presence or absence of 10 μM Ca^{2+} with or without 3 mM ATP. Values are means ± S.E.

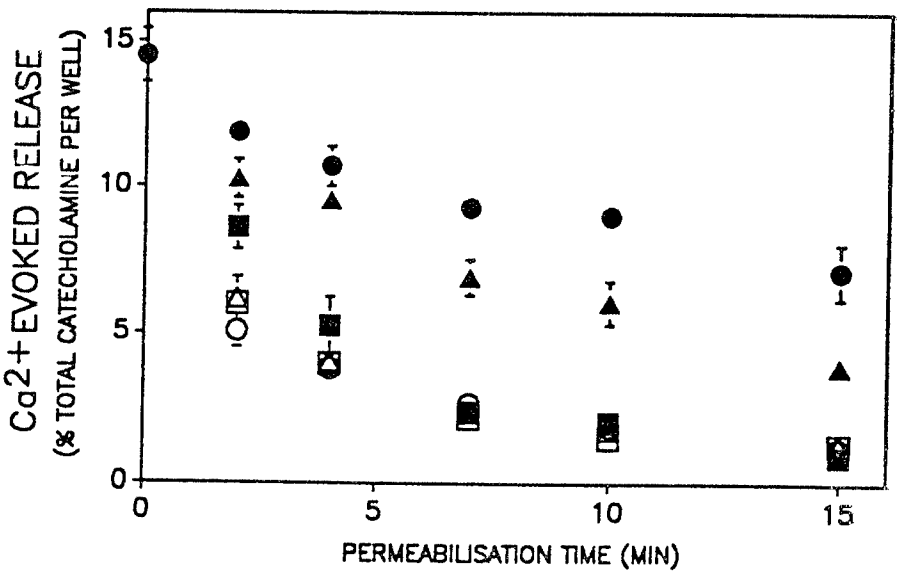


Fig. 25. BoNT/A and LC of TeTX inhibit the MgATP-dependent but not the independent component of Ca^{2+}-evoked catecholamine release from digitonin-permeabilised adrenochromaffin cells. (A) Chromaffin cells were incubated with 20 μM digitonin in KGEP (permeabilisation medium) for the indicated times in the presence (▲●■) or absence (○△□) of 2 mM MgATP. In concurrent incubations, 100 nM BoNT/A (△▲) or 100 nM TETX LC (□■) were included during the permeabilisation step. The cells were then transferred to KGEP, with or without MgATP and 10 μM Ca^{2+} (for stimulation); some wells were exposed to KGEP without Ca^{2+} to record basal release and this value was subtracted from that in the presence of Ca^{2+} to calculate the Ca^{2+}-evoked release. After 15 min the supernatants were assayed for catecholamine content, as outlined in Methods [2 (v)].

Dilution of anti-BoNT/A LC antibody	Reaction of BoNT/A gold-conjugate with anti-BoNT/A LC antibody with various ratios of BoNT/A to gold colloid used for conjugation (µg BoNT/A per ml gold colloid)									
	0	0.25	0.5	1	2	4	8	16	32	65
neat	+	+	++	++	++	++	++	++	+	+
1:10	-	+	++	++	++	++	++	++	+	+
1:100	-	-	++	++	++	++	++	++	+	+
1:1000	-	-	-	+	+	+	++	+	-	-

-, negative; +, and ++, intensity of spot detected after silver enhancement

Table 1. Measurement of the optimisation of conjugation of BoNT/A to colloidal gold. The optimum ratio of protein to gold colloid for conjugation was determined by adding increasing amounts of BoNT/A (0-65 µg) to 1 ml colloidal gold, as described in the Methods [section 2(i)]. Conjugate was incubated with nitrocellulose strips onto which a diluted series of anti-BoNT/A antibody had been dotted from a 1 µl aliquot. Bound gold was visualised as brown/black spots after silver enhancement of the strips.

	THIOL CONTENT (mol/mol protein)	MOUSE TOXICITY (LD_{50}/mg protein)
BoNT/A	5.6 ± 0.6 (5)	2×10^8
Red-A-BoNT/A	0.6 ± 0.2 (0)	6×10^4 - 2.5×10^5
Red-A-BoNT/A (reduced)[a]	0.4 ± 0.3 (0)	-

Table 2. Thiol contents and toxicities of native and Red-A-BoNT/A. These were evaluated as described in the text and are presented as means ± S.D. (n > 6). The thiol contents in parenthesis represent the expected values as collated from published sequence data (Binz *et al.*, 1990; Thompson *et al.*, 1990; DasGupta, 1981). [a] Samples further reduced with 100 mM DTT (60min / 37°C) before thiol evaluation. Adapted from **Ashton *et al.* (1993)**.

	THIOL CONTENT (mol/mol protein)	MOUSE TOXICITY (LD_{50}/mg protein)
Alk-BoNT/A	0.7 ± 0.4 (0)	2×10^8
Alk-BoNT/A (reduced)[a]	4.5 ± 0.5 (4)	-
HC	4.3 ± 0.4 (4)	-
Ren-A-HC	0.2 ± 0.1 (0)	-
Ren-A-HC (reduced)[a]	2.7 ± 0.2 (2)	-
LC	2.9 ± 0.3 (3)	-
Alk-LC	0.3 ± 0.1 (0)	-

Table 3. Sulphydryl content and mouse toxicities of A-BoNT/A, HC and LC plus their alkylated derivatives. See legend to Table 2. Adapted from **Ashton *et al.* (1993)**.

mAb	IgG class	ELISA Apparent titres[1] (Dilution fold)			Western Blot Relative reactivity (dilution fold)		Detection limit dot blot assay Apparent titres [2] (dilution fold)		Immunoprecipitation (% ^{125}I-BoNT, LC sedimented) (dilution fold)	
		BoNT	LC	LC peptide	LC	BoNT	LC	LC peptide	^{125}I-BoNT [3]	^{125}I-LC [4]
mAb 1	IgG1	$2.5x10^4$	$2.5x10^4$	nil	10^3	$2.5x10^5$	10^4	nil	10 ($2.5x10^2$)	Not determined
mAb 2	IgG1	$5X10^5$	10^6	nil	$>10^4$	$>10^6$	$>10^6$	nil	19 (10^2)	Not determined
mAb 3	IgG1	10^4	$5x10^4$	nil	10^3	$>10^6$	$>10^6$	nil	32 (10^2)	Not determined
mAb 4	IgG1	$5x10^4$	10^2	nil	10^2	$>10^6$	$2.5x10^5$	nil	86 ($5x10^2$)	58 ($2.5x10^3$)
mAb 5	IgG1	10^5	10^2	nil	10^3	$2.5x10^5$	$2.5x10^5$	nil	63 ($5x10^2$)	55 ($2.5x10^3$)
mAb 6	IgG1	10^4	10^2	nil	$<10^2$	$2.5x10^4$	10^3	nil	43 ($2.5x10^2$)	39 ($2.5x10^2$)
mAb 7	IgG 2b	$2.5x10^4$	$5x10^4$	$5x10^4$	$>10^4$	$2.5x10^5$	$2.5x10^5$	10^5	10 (10^2)	5 (10^2)
Mouse[5] serum	Not relevant	10^5	10^5	$<10^2$	$>10^5$	$5x10^5$	$>10^6$	$<10^2$	107 (10^4)	103 (10^4)

Table 4. Characteristics of mAbs ascitic fluids raised to LC epitopes of type A BoNT. [1] Apparent titres expressed as the dilution of mAb giving a unit of absorbance ≥ 1.0. [2] Maximum dilution detectable. [3] Dilution (fold) precipitating maximal % of specifically-bound ^{125}I-BoNT. [4] Dilution (fold) precipitating maximal % of specifically-bound ^{125}I-LC. [5] Sera collected from hyperimmunised mice whose spleen were used in fusions. Taken from **Cenci di Bello *et al.* (1993).**

Inhibitor	Final conc.	% Inhibition	
		BoNT/B	TeTX
EDTA	1 mM (pre 5 mM)	>95%	>95%
PTL	1 mM (pre 5 mM)	>95%	>95%
PTL treated + Zn^{2+} replaced		14%	20%
Captopril	2 mM (pre 20 mM)	0%	90%
ASQFETS	2 mM (pre 10 mM)	0%	45%
Phosphoramidon	0.5 mM (pre 2.5 mM)	0%	0%

Table 5. Differential effects of inhibitors on proteolysis of Sbr in salt-washed SCVs by BoNT/B and TeTX. Each toxin was pre-incubated for 45 min at 37°C with 40 mM DTT, in the absence or presence of inhibitors (designated pre) and added to a suspension of rat SCVs (0.25 mg/ml) at a final concentration of 100 nM. The incubations were continued for 90 min at 37°C, followed by solubilisation in SDS-PAGE sample buffer, electrophoretic transfer to PVDF and Western blotting for Sbr and synaptophysin. The amounts of Sbr remaining after incubation were quantified by densitometric scanning using synaptophysin as an internal standard.