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Neurotransmitter release is blocked intracellularly by botulinum neurotoxin, and this requires uptake of both toxin polypeptides by a process mediated by the larger chain

(cholinergic synapse/noncholinergic synapse/intracellular injection/quantal release/*Aplysia californica*)

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ABSTRACT **Botulinum neurotoxins (types A and B), which are microbial proteins consisting of two disulfide-linked chains, inhibit specifically and with high potency the release of acetylcholine from peripheral nerve terminals. As a prerequisite for a long-term development of effective treatments for botulism, the internalization and inhibitory action of the toxin and its constituent chains were examined by electrophysiological methods at identified synapses in *Aplysia* preparations that allow both intracellular and bath application of the neurotoxins. Intracellular recordings from cholinergic cells of the buccal ganglion demonstrated that extra- or intracellular application of low doses of botulinum neurotoxin results in a specific blockade of evoked transmitter release, without changing the quantal size; an intraneuronal site of action has thus been established. In contrast, release from noncholinergic neurons of cerebral ganglion was prevented by the neurotoxin only after injection into the cell. Purified preparations of the individual renatured chains, shown to be nontoxic in a mouse bioassay, failed to affect acetylcholine release when applied extra- or intracellularly. However, inhibition of release was observed after intracellular administration of both chains or when the light chain was injected and the heavy chain was bath-applied. These findings show that both chains are required on the cytosolic side of the neuronal plasma membrane for expression of toxicity and that the cholinergic specificity of the neurotoxin is attributable to its heavy chain, which mediates targeting and subsequent neuronal uptake.**

Botulinum neurotoxin (BoNT) types A and B, highly neurotoxic proteins ($M_r \approx 150{,}000$) produced by strains of *Clostridium botulinum*, contain a heavy chain (HC; $M_r \approx 95{,}000$) and a light chain (LC; $M_r \approx 55{,}000$) linked by at least one interchain disulfide bond (1). Minute quantities of these proteins cause neuroparalysis (LD_{50} in mice, 12 pg) by a preferential and irreversible blockage of acetylcholine (AcCho) release in the peripheral nervous system (2, 3). At present there is no effective treatment available for this fatal condition, botulism. Deciphering the basis of this potent action of BoNT might yield an eventual cure, as well as give insight into the mechanism of transmitter release. Moreover, such information should improve the use of the toxin in treating patients suffering from neuromuscular disorders such as blepharospasm and strabismus (4).

The cholinergic specificity of BoNT, which has provided its clinical application, has been attributed to receptors present on the terminal membrane of cholinergic nerves (5, 6). These bind the toxin and mediate its internalization at motor nerve terminals by an energy-dependent, temperature-sensitive mechanism (7, 8). However, direct evidence is lacking for a multistep process of intoxication (2, 9) and, in particular, for the inactivation by BoNT of an intracellular component that is involved in the release of AcCho and, perhaps, all transmitters. Furthermore, the role of the HC and LC of BoNT in such a scheme for its neuroparalytic action remains to be established. Pertinent questions to be answered include (*i*) whether the HC, by virtue of its ability to recognize neural membrane receptors (8, 10), allows functional recognition of cholinergic terminals and whether it can act as a transport vehicle for the LC and (*ii*) which chain(s) gives rise to the postulated intracellular poisoning event. Thus, the aim of the present study was to demonstrate an intraneuronal inhibitory action on nerve-evoked release of transmitters in different neuron types in *Aplysia*, when BoNT or its individual chains were applied inside or outside the cells.

MATERIALS AND METHODS

Purification and Characterization of BoNT and its Chains. BoNT type A (11) and B (12), purified to homogeneity and with respective specific neurotoxicities (after trypsinization) of 2×10^8 and 1×10^8 mouse median lethal doses (LD_{50}) per mg of protein, was dialyzed into artificial sea water (ASW: 460 mM NaCl/10 mM KCl/11 mM $CaCl_2$/25 mM $MgCl_2$/28 mM $MgSO_4$/10 mM Tris·HCl, pH 7.8). To prepare HC and LC, any single-chain form of BoNT A or B present was cleaved into the two chains by limited proteolysis. Type A was treated with protease Arg-C (20 units/ml) at 37°C for 20 min, whereas type B was incubated with trypsin (5 μg/ml) at 22°C for 20 min; digestion was arrested by the addition of L-7-amino-1-chloro-3-tosylamido-2-heptanone ("tosyl-L-lysine chloromethyl ketone," 2 mM) or soybean trypsin inhibitor (10-fold molar excess), respectively. This was followed immediately by FPLC chromatography using a Mono Q anion-exchange column (Pharmacia, Uppsala, Sweden) to separate the two-chain form of each toxin from the protease inhibitor. HC and LC of types A and B BoNT were then isolated by adsorption to QAE-Sephadex, followed by addition of dithiothreitol and urea to remove the LC in the wash buffer; HC was subsequently eluted by 0.2 M NaCl (13, 14). The polypeptides from BoNT A and B were renatured separately at 4°C by sequential dialysis (without agitation) into 10 mM Tris·HCl, pH 7.8/0.46 M NaCl and ASW. Their biological activities were assessed by intraperitoneal injection into mice and by measuring twitch tension of a mouse phrenic-nerve/diaphragm preparation after bath application of each chain. As the ultimate criterion of activity, the

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Abbreviations: BoNT, botulinum neurotoxin; LC, light chain; HC, heavy chain; AcCho, acetylcholine; MPSC, miniature postsynaptic current; LDIPSC, long-duration inhibitory postsynaptic current; ASW, artificial sea water.

two-chain form of BoNT was reconstituted (as monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis) by mixing equimolar amounts of HC and LC from type A or B and dialyzing the mixtures at 4°C over 4 days into 50 mM Tris·HCl buffer (pH 7.7), to remove the urea and reducing agent. The two resultant samples had specific neurotoxicities in mice equivalent to ≈20% of the respective native toxin, and both of the samples blocked neuromuscular transmission in mouse diaphragm.

Electrophysiological Recording of the Action of BoNT and its Chains on the Release of Transmitters. Experiments were performed with preparations from *Aplysia californica* (Pacific Biomarine, Venice, CA). Recordings were made from identified couples of cholinergic neurons in the buccal ganglia, which make a well-known chloride-dependent inhibitory synapse (15–17), and on noncholinergic neurons of cluster A in the cerebral ganglion, which make cationic excitatory synapses on neurons of the cluster B (18). The neurotransmitter, definitely not AcCho, has not been identified (18). Dissected ganglia were pinned in a 1-ml chamber and superfused (10 ml/hr) at room temperature (23°C) with ASW except when tetrodotoxin, BoNT, or isolated chains were added to the bath.

Neurotransmitter release was measured as a function of the amplitude of the postsynaptic response; both pre- and postsynaptic cell bodies were impaled by two micropipettes (3 M KCl, 1–10 MΩ). Postsynaptic responses due to the neurotransmitter release evoked by an action potential in the presynaptic neuron were recorded as voltage or current changes by current- and voltage-clamp techniques. Current values were then expressed as conductance.

At the cholinergic synapse in the buccal ganglion, the size of AcCho quanta was calculated from evoked postsynaptic responses. The close proximity (300–500 μm) of the cholinergic nerve terminals to the postsynaptic cell body allowed simultaneous voltage clamp of both pre- and postsynaptic neurons. In the presence of bath-applied tetrodotoxin (0.1 mM) to block action potentials, it was possible to induce the release of AcCho by a sustained (3-sec) depolarization of the presynaptic neuron. The resulting postsynaptic response, termed long-duration inhibitory postsynaptic current (LDIPSC), showed fluctuations due to summation of evoked miniature postsynaptic currents (MPSCs). Statistical analysis of the LDIPSCs permitted calculation of their amplitude and duration and, hence, of the quantal content of the LDIPSC (16). This technique could not be used on noncholinergic synapses, which are unable to produce a sustained release of the transmitter.

Solutions of BoNT or isolated chains, to which a dye (fast green FCF, Sigma) was added in order to visualize the injection, were air-pressure-injected into a presynaptic neuron by using a third micropipette, which was removed after injection (15, 17). Intracellular injections of control solutions (plus added dye) were shown to have no effect on neurotransmitter release; these represented the various buffers used to prepare the neurotoxin samples, which were dialyzed similarly against ASW.

RESULTS

Characterization of the Inhibitory Effects of Bath-Applied BoNT on AcCho Release. The action of BoNT, applied extracellularly, on cholinergic transmission was examined at concentrations (1–10 nM) known to be effective *in vitro* on transmitter release in vertebrate preparations [motor end plates (19), autonomic nerves (20), brain synaptosomes (3), and *Torpedo* electric organ (21)]. Bath application of type A (Fig. 1, ▲) or B to the buccal ganglion gave a time- and concentration-dependent depression of evoked quantal release of AcCho. This inhibition was seen when release of the

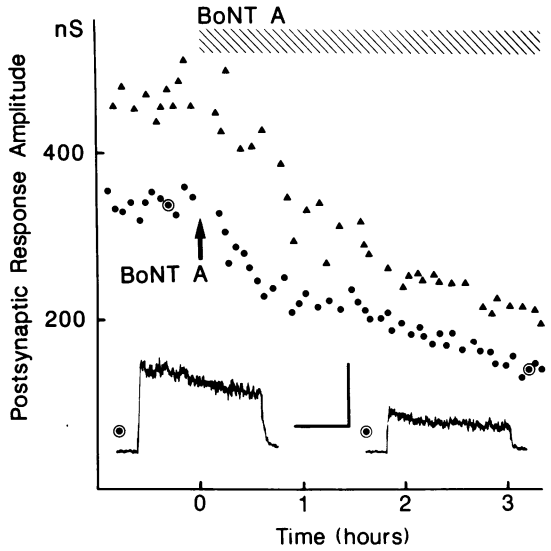

FIG. 1. Decrease in AcCho release induced by BoNT type A. AcCho release was evoked at an identified cholinergic synapse in the buccal ganglia in *Aplysia* by a 3-sec depolarization (to +10 mV) of the presynaptic neuron under voltage clamp. The amplitudes of LDIPSCs recorded at 23°C in the voltage-clamped (−80 mV) postsynaptic neurons are expressed as conductance (nS). After control recordings, BoNT type A was bath-applied at 7 nM (hatched bar, ▲) or, in another preparation, air-pressure-injected into the cell body of the presynaptic neuron (arrow, ●) to give a calculated intracellular concentration of 5 nM. (*Insets*) Postsynaptic recordings at the point indicated in the control conditions (left) and during the decrease in AcCho release (right) resulting from the intraneuronal administration of BoNT. (Vertical bar = 300 nS; horizontal bar = 1.2 sec.) In the reported experiments, the calculated MPSC amplitudes were 0.99 ± 0.23 nS for control and 1.05 ± 0.17 nS after 3 hr of bath application of BoNT and 1.55 ± 0.20 nS for control and 1.62 ± 0.25 nS 3 hr after injection of BoNT. Each value of MPSC was calculated from at least five subsequent recordings. All parts of the experiments have been repeated on at least three separate preparations, and the same pattern of blockade with BoNT was observed.

transmitter was evoked by a presynaptic action potential (see below) or induced by long-duration depolarization of the presynaptic neuron (Fig. 1), the method used to analyze the quantal aspects of transmission. Clearly, the decrease in AcCho release could not be due to alteration of action-potential propagation or size, in accord with observations made at the mouse neuromuscular junction (22). Also, the blockade cannot be attributed to the prolonged stimulation used, as AcCho release was not reduced in the absence of BoNT (17). The depression could be observed in the absence of stimulation or when spontaneous activity was suppressed by tetrodotoxin; however, preliminary results showed that an intense stimulation induced an acceleration in the rate of decrease of AcCho release, suggesting a relationship between BoNT action and neuronal activity. Apparently, irreversible binding and/or sequestration of BoNT occurred during the 1 hr of exposure in the bath, because the rate of decrease in the postsynaptic response did not change after toxin removal at this time. As in the case of vertebrate motor nerve terminals (9, 23, 24), the toxin's action was temperature-dependent. When the experiment was performed at 8–10°C, no significant decrease in the postsynaptic response was observed within 2 hr of bath-application of 7 nM BoNT A; however, when the temperature was raised to 23°C, a decrease in AcCho release ensued.

Statistical analysis of LDIPSCs revealed that BoNT-induced inhibition of AcCho release was due solely to a decrease in the number of quanta released; the calculated MPSC amplitude, and thus the size of AcCho quanta, remained unchanged (see legend to Fig. 1). These numerous

similarities in the measurable effects of BoNT at the vertebrate neuromuscular junction and on *Aplysia* neurons (see also below) validate the use of the *Aplysia* system to gain insight into the molecular basis of the toxin's action in vertebrates.

When Applied Intracellularly, BoNT Is a Powerful Inhibitor of AcCho Release. As it has been suggested that all or part of BoNT has to enter the nerve terminal to exert an action (5), this possibility was tested by injecting BoNT into a presynaptic cholinergic neuron. This gave rise to a depression of the postsynaptic response (Fig. 1, ●); a 50% decrease was observed within 1–4 hr, depending of the quantity of BoNT injected, with nearly complete blockade after 24 hr. As in the case of bath-applied BoNT, inhibition resulted only from a decrease in the number of quanta released (see legend to Fig. 1); likewise, no modification resulted in the presynaptic action potential. Although there are practical difficulties in measuring precisely the volume of BoNT injected, it was estimated to be about 1% of the volume of the neuron; thus, the intracellular concentration of BoNT A and B applied ranged from 1 nM to 10 nM in the soma. When the diffusion distance (300–500 μm) between the cell body and the terminal is taken into account, the relatively rapid onset of the depression suggests that very low concentrations of BoNT are required intracellularly to abolish the functioning of a component concerned with AcCho release.

BoNT Is an Equipotent Intracellular Blocker of Release from Noncholinergic Neurons. The effect of BoNT was assessed on identified and characterized noncholinergic neurons in the cerebral ganglion (18). In contrast to the cholinergic neurons in the buccal ganglion, neuronal stimulation by action potentials evoked repeatedly in the course of the experiments can induce a slight decrease in transmitter release (Fig. 2, ○). Bath-applied BoNT A (7 nM) yielded no further reduction in transmitter release (data not shown), possibly due to the absence of acceptor-mediated uptake of the neurotoxin. However, when the internalization step was bypassed by an intracellular injection of BoNT A (Fig. 2, ●), a pronounced reduction in transmitter release was observed, relative to an equivalent noninjected neuron afferent to the same postsynaptic cell.

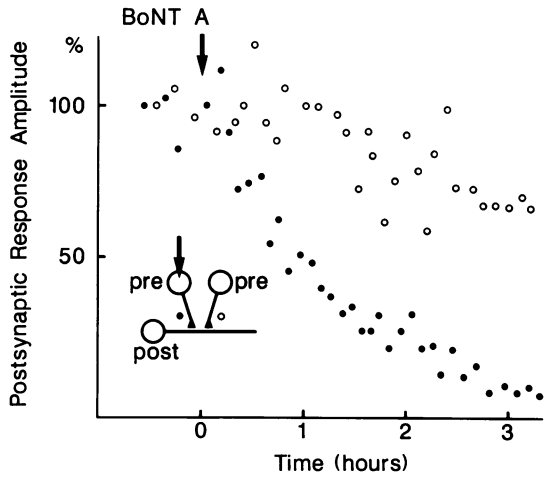

FIG. 2. Inhibition of transmitter release in a noncholinergic neuron induced by intracellular injection of BoNT. Transmitter release was evoked at a noncholinergic synapse in the cerebral ganglia of *Aplysia* by a presynaptic action potential; the resultant postsynaptic responses were recorded as membrane potentials. In contrast to the buccal ganglion, repetitive activation of this synapse modifies its efficacy slightly. Consequently, the changes (%) in amplitude of the postsynaptic response (●) after an injection of BoNT type A (arrow) into the presynaptic neuron were compared with the responses (○) of noninjected equivalent neuron, afferent to the same postsynaptic cell and releasing the same neurotransmitter (a schematic drawing of the synaptic connections is shown). The control neuron and the injected one were stimulated alternately. Calibration: 100% = 1.8 mV (●) or 1.5 mV (○).

Expression of Toxicity Requires the Presence Within the Neuron of Both the LC and the HC of BoNT. When pure LC and HC of types A and B were renatured by stepwise dialysis into ASW, the individual chains showed little residual toxicity in the mouse bioassay ($<10^3$ mouse LD_{50}/mg) and, accordingly, were found to be ineffective on neuromuscular transmission when bath-applied individually at 30 nM to mouse diaphragm for 6 hr. Bath application of LC or HC (up to 125 nM) did not induce any change in transmitter release at the cholinergic synapse in *Aplysia*. Likewise, injection of either chain into the presynaptic neuron (concentration within the soma estimated to be 1–2.5 nM) resulted in no modification in AcCho release. However, injection of a similar quantity of an equimolar mixture of LC and HC produced a decrease in AcCho release (Fig. 3 *Top*) that was similar to that elicited by the native BoNT (*cf.* Fig. 1). When the two chains were injected one after the other (for instance, 150 min apart), the diminution in AcCho release started only when the second chain was introduced inside the neuron (data not shown). Moreover, bath application of HC (20 nM) to a preparation in which the presynaptic cell had been injected previously with the LC initiated a decrease in AcCho release (Fig. 3 *Middle*). Subsequent removal of HC from the bath by washing within 2 hr of its addition did not induce a recovery (data not shown). Injection of HC into the presynaptic cell when a high concentration (100 nM) of LC was bath-applied proved ineffective. Notably, the gross effects on AcCho release of nicked preparations of the two neurotoxin types (A and B) examined were not distinguishable. Indeed, the chimeric toxin (the LC from either type and the HC of the other) induced a decrease in AcCho release when applied within the neuron, or when the LC was injected and the HC was bath-applied (Fig. 3 *Bottom*).

DISCUSSION

Until now, BoNT inhibition of *quantal* release of transmitter has been demonstrated at peripheral nerves of vertebrates only. The results presented here show not only that the neurotoxin blocks evoked release in invertebrate ganglion preparations but also that its effects are indistinguishable from those reported for vertebrate tissues. For example, consistent with the reported preferential sensitivity of mammalian cholinergic synapses to BoNT A (20), evoked transmitter release from a noncholinergic neuron proved insensitive when neurotoxin was added to the bath at the concentrations used in the present study. As in the case of murine peripheral nerves (5, 6), this might be attributable to the presence of surface receptors on cholinergic neurons only; however, the direct demonstration of such receptors in *Aplysia* cells remains to be obtained.

An important outcome of this investigation, relating to the postulated multiphasic action of BoNT, was the clear demonstration that evoked release from both cholinergic and noncholinergic neurons is decreased by intracellular administration of the neurotoxin. This establishes conclusively that BoNT acts within the neuron on a component part of the release process that is common (5) to a number of transmitters. This toxin-sensitive site seems fairly ubiquitous, as secretion from adrenal chromaffin cells is blocked by BoNT applied intracellularly (25) or by prolonged (>5 days) exposure of the cells in culture (26). The neurotoxin blocks (albeit at high concentration) the efflux from brain synaptosomal preparations of AcCho, dopamine, noradrenaline and γ-aminobutyrate (3); glutamate (27); and glycine and enkephalin (28). Presumably, at high concentration or during prolonged exposure, the neurotoxin enters by a receptor-

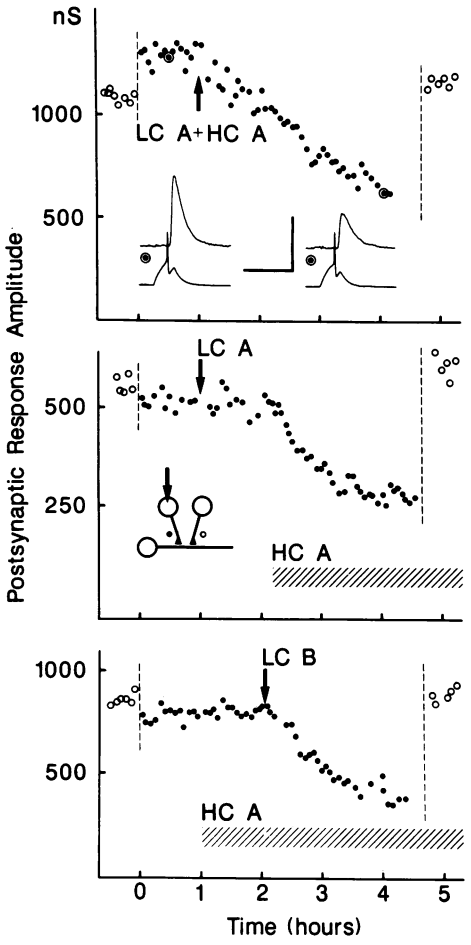

FIG. 3. Both LC and HC are required intracellularly for blockade of transmitter release. In all experiments performed with the chains of BoNT, AcCho release was evoked by presynaptic action potential; responses were recorded in the voltage-clamped postsynaptic neuron and are expressed as membrane conductance (nS). Changes in the transmitter release of the injected cell (●) were compared with those of a second, noninjected presynaptic neuron (○) afferent to the same postsynaptic cell (*Inset* in *Middle* is a schematic drawing of synaptic connections in the buccal ganglion). In all cases, the responses to the control neuron remained unmodified. (*Top*) After representative control measurements were made, equimolar amounts of LC and HC of type A were mixed and immediately injected (arrow) into the presynaptic neuron. (*Inset*) Actual records of the postsynaptic response induced by an action potential at time indicated (◉) before (left) and after (right) injection. (Vertical bar = 80 mV for the presynaptic action potential and 1000 nS for the postsynaptic response; horizontal bar = 75 msec.) (*Middle*) Injection (arrow) of type A LC did not diminish AcCho release, but addition to the bath of type A HC (hatched bar) at 20 nM led to the expression of toxicity. (*Bottom*) Bath application (hatched bar) of type A HC at 20 nM did not affect AcCho release, but injection (arrow) of type B LC led to the normal decrease in AcCho release. Thus, the chimeric A–B toxin is active.

independent route, since noncholinergic neurons have been shown to be devoid of detectable binding sites (5, 6).

Since intracellular injection of native toxin resulted in a reduction in the release of transmitters, toxicity can be expressed independently of binding to receptors and the ensuing translocation process; this implies that processing of the molecule during transport may not be essential. Contrary to the situation reported for diphtheria toxin where the lighter chain alone can inhibit protein synthesis in a cell-free system (29), the presence inside the neuron of both the LC and the HC of BoNT is required for the observed blockade of transmitter release. The toxicity produced by sequential application of LC and HC of BoNT inside the cholinergic neuron raises the interesting question of whether these two proteins exert their effect at distinct loci by a concerted mechanism or whether they associate and act jointly. On the other hand, the extracellular role(s) played by the HC was deciphered: when HC and LC were applied simultaneously on opposite sides of the plasma membrane, toxicity was seen only when it was the HC that was applied outside the neuron. This implies that the HC alone can presumably interact with surface receptors and that it can be translocated on its own or when associated with LC. It is noteworthy that chimeric forms of the neurotoxin (LC and HC from types A or B) are active inside the cell or when LC of one type is placed inside and HC of the other is bath-applied.

In conclusion, *Aplysia* neurons have been shown to be appropriate models for study of BoNT; also, the preparations offer the advantage of allowing accessibility to either side of the membrane of one neuron. BoNT blockade of cholinergic transmission requires internalization of both HC and LC, the intracellular target being common to cholinergic and noncholinergic cells. The characterization of such an important and widespread component is now feasible by use of the appropriate fragment of BoNT. A practical possibility arising from these findings includes the use of a nontoxic derivative of HC to target neutralizing antibodies into affected nerves of patients suffering from botulism.

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