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Light chain of tetanus toxin intracellularly inhibits acetylcholine release at neuro-neuronal synapses, and its internalization is mediated by heavy chain

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The ability of the two-chain form of tetanus toxin (TeTx), its constituent light (LC) or heavy (HC) chains, and papain fragment to block evoked acetylcholine (ACh) release in the buccal ganglia of *Aplysia californica* was studied electrophysiologically. Extracellularly applied, TeTx or its B fragment (consisting of LC and β_2, the amino-terminal portion of HC) blocked ACh release, whereas LC, HC, or the β_2 fragment did not affect it. Toxicity was restored when LC was bath applied together with HC or the β_2 fragment. When injected into the presynaptic neuron, TeTx, the B fragment or LC, but not HC, induced inhibition of ACh release. These results indicate that the blockade of ACh release by TeTx is mimicked by intracellular action of LC, the internalization of which is mediated by the HC via its amino-terminal moiety.

Tetanus toxin; Light chain; Heavy chain; Transmitter release; Central synapse; (*Aplysia*)

1. INTRODUCTION

Tetanus toxin (TeTx) is known to block the transmitter release from central nerve terminals and, at much higher concentration, acetylcholine (ACh) release from motor end-plates in vertebrates (for review see [1,2]). TeTx, produced in culture broth of *Clostridium tetani*, is a two-chain protein consisting of heavy (HC, 98 kDa) and light (LC, 52 kDa) chains linked by a disulfide bond [3]. Recent work on non-neuronal preparations indicates that the toxic site for blocking of the transmitter release exists on the LC of TeTx. Catecholamine release was blocked in bovine adrenal medullary chromaffin cells permeabilized with streptolysin-O to allow internalization of LC [4], and intracellular injection of the B fragment of TeTx (consisting of β_2 the amino-terminal moiety of the HC which is linked to LC by a di-sulfide bond) blocked capacitance changes associated with the catecholamine release [5]. These findings contrast with the demonstration [6–8], in a neuro-neuronal cholinergic synapse of *Aplysia*, of the intracellular requirement of both LC and HC for the blocking action of botulinum neurotoxins (BoNTs). These toxins exhibit a gross structure similar to TeTx [1,2]. It seemed thus interesting to assess whether and how TeTx and its chains act on *Aplysia* synapses. These toxins can be injected into the presynaptic neurons and the release of the neurotransmitter is directly related to the postsynaptic response.

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2. EXPERIMENTAL

2.1. *Materials*

The two-chain form of tetanus toxin was prepared either by chymotrypsin cleavage of the single-chain form of the toxin (TeTx BC) or from culture filtrate (TeTx BE) according to a previous procedure [9]. Their constituent HC and LC were obtained by isoelectric focussing in a sucrose gradient with am-

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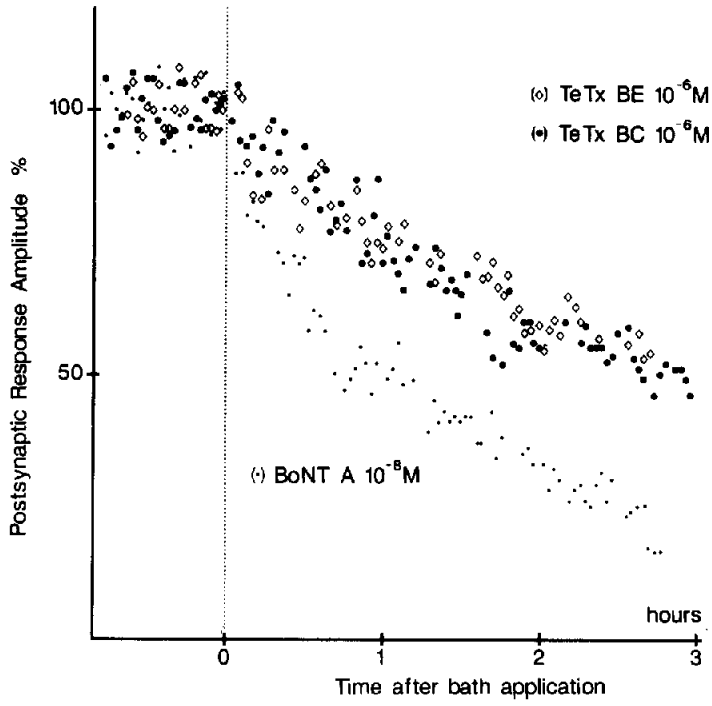

Fig.1. Effect of bath applied tetanus toxin (TeTx) on a cholinergic synapse of *Aplysia*. Postsynaptic responses evoked by the presynaptic action potential (once a minute) were recorded against time. Typical experiments using three distinct preparations are reported. After a period of stabilization in synaptic responses (100%), the two-chain form TeTx BE or TeTx BC (10^{-6} M) was bath applied at time 0. For comparison, the depression of synaptic transmission induced by the botulinum toxin type A (BoNT A) at 10^{-8} M is shown.

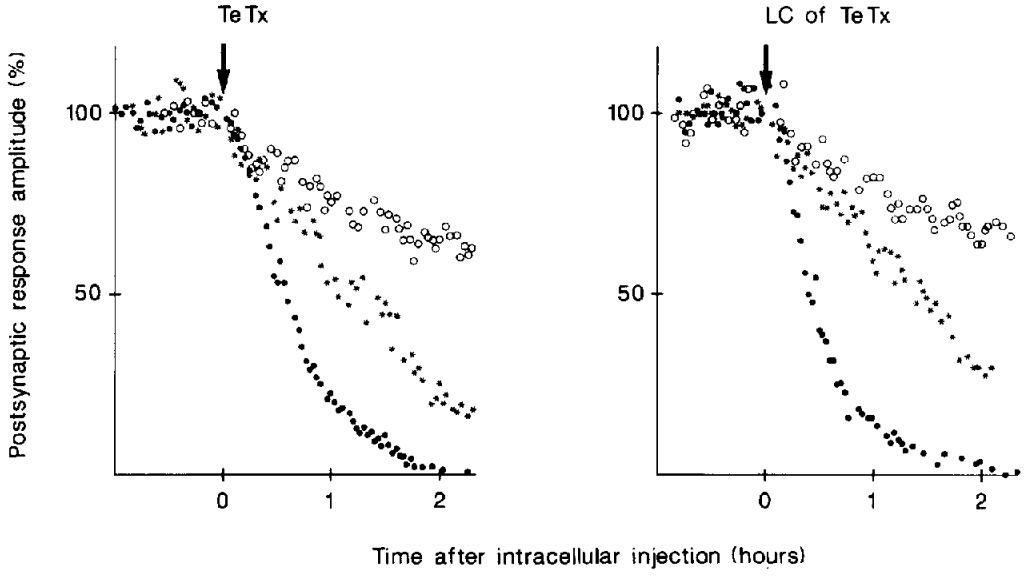

Fig.2. Intracellularly applied TeTx or TeTx LC depress the release of ACh. The two-chain form TeTx (BC) or isolated LC was injected (arrow) into a presynaptic neuron. Concentrations of TeTx or LC in the micropipette used for the injection were (•) 2×10^{-5} M, (*) 2×10^{-6} M and (○) 2×10^{-7} M leading to theoretical intracellular concentrations in the range of 2×10^{-7} M, 2×10^{-8} M and 2×10^{-9} M, respectively, assuming that the injected volume was about 1% of cell body. Note that the potency of LC to block ACh release is the same as that of TeTx.

pholyte under reducing conditions in 2 mol/l urea. Toxicity (LD_{50}, s.c. in mice) was 2 ng/kg for TeTx BE or BC, 100–300 μg/kg for both HC and LC. The B fragment of tetanus toxin obtained by papain cleavage [10,11] exhibited a toxicity of 5–50 μg/kg. Following reduction of the latter fragment, the β_2 fragment was isolated by isoelectric focussing [10]. All toxin prepartions were dialysed 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), prior to use. Highly purified botulinum neurotoxin type A (BoNT A) was a gift from Dr J.O. Dolly, Dept of Neurochemistry, Imperial College, London (England).

2.2. *Electrophysiological recordings*

Experiments were made in *Aplysia californica* on identified cholinergic neuro-neuronal synapses in the buccal ganglion [12] and on synapses in the cerebral ganglia [13] that release a not yet identified non-cholinergic transmitter [14]. Experimental procedures were similar to those used for the study of BoNT [6–8]. Briefly, dissected ganglia were pinned in a 1 ml chamber and superfused (10 ml/h) at room temperature (22–23°C) with ASW, except when toxin, chains or fragments were added to the bath. Both pre- and postsynaptic neurons were impaled with two glass micropipettes (3 M KCl) for current- or voltage-clamp measurements. ACh release was reflected by the amplitude of the postsynaptic current response elicited by the evoked action potential in the presynaptic neuron. The action potential was not affected by any toxin, chains or fragments. Intracellular application of toxin, chain or the fragment B was achieved by an air pressure injection through a third micropipette [6–8,12].

3. RESULTS

Extracellular application of the two-chain form of tetanus toxin to the cholinergic synapse in the buccal ganglion of *Aplysia* induced a time-dependent and irreversible depression of transmission (fig.1). At 10^{-6} M, 50% decrease in the amplitude of evoked postsynaptic responses was observed within 3 h. The two-chain forms of TeTx (BE and BC) were equipotent. By comparison to the action of BoNT A, TeTx is more than 100-fold less potent (fig.1). Bath addition of TeTx (10^{-6} M) to the BoNT-resistant [6] non-cholinergic synapses in the cerebral ganglia [13,14] induced a rapid decrease in transmission (50% blockade after 25 min, not shown). This suggests that the lower potency of TeTx applied extracellularly to depress the cholinergic terminal is due to a weak internalization of the toxin. Accordingly, when this step was by-passed by an intracellular injection of TeTx, a time-dependent inhibition of ACh release was induced even by a low concentration of TeTx (fig.2A): note that a complete blockade was observed within 2 h for an estimated intracellular concentration in the 10^{-7} M range.

Intracellularly injected LC alone depressed ACh release with a potency equivalent to that of intracellularly applied TeTx (fig.2). Injected fragment B was as effective as LC. In contrast, injected HC alone was not toxic. When added to the bath alone, neither LC nor HC affected ACh release (fig.3). However, an irreversible decrease of ACh

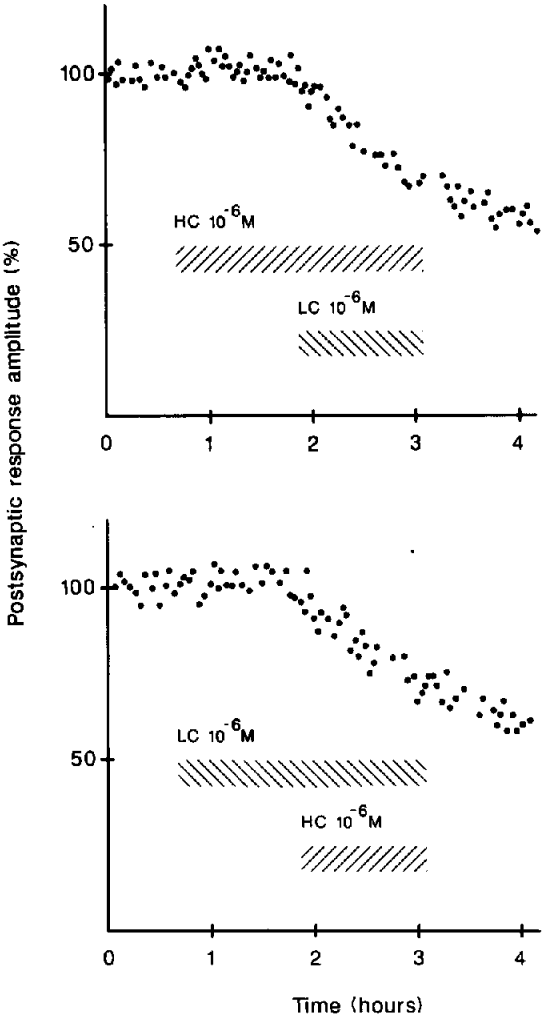

Fig.3. Depression of ACh release by extracellular application of TeTx. The presence of both HC and LC is required. Bath application (first hatched areas) of isolated chains of TeTx (10^{-6} M HC, upper graph or 10^{-6} M LC, lower graph) did not modify synaptic transmission. Upon addition (second hatched area) of the other chain (LC or HC, 10^{-6} M each, respectively) blockade of transmission ensued to an extent similar to that observed with TeTx (see fig.1).

release was observed when both chains were present together in the bath (fig.3). The extent of this decrease was similar to that induced by the same concentration of the two-chain TeTx (cf. with fig.1). Thus it appears that LC contains site(s) leading to the depression of transmitter release when inside the neuron, whereas its uptake from outside requires the presence of HC.

Since the amino-terminal moiety of the HC is involved in the internalization of BoNT in *Aplysia* [8], we tested this possibility for TeTx too. Extracellularly applied fragment B (10^{-6} M) of TeTx induced a similar depression of ACh release to that induced by the same concentration of TeTx BE or BC (not shown). When its constituent, polypeptides β_2 (that was not toxic alone) and LC were applied simultaneously in the bath (each at 10^{-6} M) a similar decrease to that induced by B fragment or TeTx ensued. This indicates that the amino-terminal moiety of the HC is involved in the internalization of TeTx into *Aplysia* neurons.

4. DISCUSSION

TeTx inhibits neurotransmitter release not only at non-cholinergic but also, with less efficacy, at cholinergic neuronal synapses in *Aplysia*. Our results are in good agreement with previous reports that TeTx blocks the release of various neurotransmitters like glycine and GABA [15], and noradrenaline [16] in rat brain and was less potent than BoNT in motor end-plate terminals [17,18]. Compared to BoNT A or B [6,7], TeTx appears less potent (more than 100-fold) on cholinergic cells of *Aplysia*, this is similar to the effect on vertebrate motor end-plates [17,18]. However when injected into the cholinergic neuron TeTx exhibited a potency as strong as that of intracellularly applied BoNT type A or B [6,7]. This indicates that the observed difference in efficacy of TeTx and BoNT results rather from dissimilarities in extracellular targetting the toxins to the terminal membrane than from quantitatively different intracellular actions.

Once within the neuron TeTx LC alone is toxic. Although BoNT and TeTx have a similar two-chain form structure [1,2], this result contrasts with the intracellular requirement of both LC and HC of BoNT A or B in cholinergic neurons of *Aplysia* [6,7]. Actually, the H_2L fragment of BoNT A, homologous to fragment B of TeTx, is not toxic intracellularly [8] whereas fragment B is.

LC can mimick the action of TeTx when applied into the cell but not to its surface, thus the chain cannot enter the neuron on its own. The internalization process needs the presence of the HC. The carrier function of HC of clostridial toxins (TeTx or BoNT) is supported by their role in binding of TeTx or BoNT to membrane acceptors [10,19–21]. Native and reconstituted TeTx BE, or HC but not LC bind to rat brain membranes [10,19]. Fragment B does not affect ACh release at the vertebrate motor end-plate [22], perhaps because here the carboxy-terminus of HC (called C fragment) of TeTx is required for binding to membrane gangliosides (see for review [1,2]). However, an additional contribution of the amino-terminus of HC was also reported for TeTx binding to rat brain membranes [10]. In *Aplysia*, as evident from the depression of ACh release induced by fragment B or a mixture of β_2 and LC, the region of HC involved in the internalization step is the amino-terminal β_2. The same role was attributed to the amino-terminal H_2 of BoNT homologous to β_2 of TeTx [8].

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